

Organic Matter Transformation by *Gammarus* in Lowland Chalk Streams

Submitted by

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Abstract

In chalk streams, *Gammarus pulex* is an abundant macroinvertebrate shredder that egests cohesive, cylindrical faecal pellets. The size and mass of faecal pellets influences their transport and utilisation in aquatic ecosystems, and are determined both by body size and diet in *Gammarus*. The number of *Gammarus* faecal pellets in sediments in the River Chess (a chalk stream and my field study site) varied over time, with the highest numbers found in Autumn and the lowest numbers found in Summer. Faecal pellet numbers did not vary spatially between different habitat types within the river.

Gammarus faecal pellets are bound together by exopolymer substances, and also initially by a peritrophic membrane. Their breakdown is complex and involves changes to the size of organic matter particles within the pellet (which become larger over time), and is primarily mediated by bacteria internal to the pellets themselves. Although changes occur to the pellet during breakdown, the overall structure of the pellet is maintained for long periods, and *Gammarus* faecal pellets are relatively stable structures which have the potential to store organic matter in the environment.

Organic materials in *Gammarus* faecal pellets are utilised by bacteria and other micro-organisms as a substrate, bacterial numbers peaking soon after pellet egestion, and microbial respiration rates of faecal pellets are maintained at relatively high rates. Invertebrates also consume *Gammarus* faecal pellets, either to gain nutrition from the microbial communities there, or directly from organic matter within the pellets.

Gammarus faecal pellets are likely to be an important ecological component of chalk streams, transforming, retaining and recycling organic matter (especially allochthonous materials) so that it can be utilised by other organisms in the ecosystem.

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1. Introduction

1.1. Detritus as a Major Ecosystem Component

Non-living organic matter, or detritus (Swift *et al.* 1979) is present in all ecosystems, both aquatic and terrestrial, in some form. Dead plant materials (dead leaves, wood), dead animal bodies, faecal matter and secretions (e.g. exopolymers, dissolved organic matter) can all be considered forms of detritus. In traditional ecological studies and models detritus was considered as a homogenous resource with little spatial and temporal variation, probably because of a bias towards organism oriented community ecology in the last 50 years of research (Moore *et al.* 2004). Detritus is now known to be an important ecosystem component and in many food webs the majority of energy flows through detrital pathways (Wetzel 1983, Odum and Biever 1984). Habitats are created, modified and degraded by the presence and/or absence of detritus (Moore *et al.* 2004), and where detritus is changed from one form to another energy is transferred to otherwise disparate parts of an ecosystem – the process of ecosystem engineering (Jones *et al.* 1994, Wotton *et al.* 1998, Wotton and Malmqvist 2001). Modern ecological studies are showing that far from being a homogenous pool of organic matter, detritus is a complex

and important part of the ecology of many environments which has many forms and roles.

1.2. Invertebrate Faecal Pellets in Aquatic Ecosystems

The faeces of many terrestrial and aquatic invertebrates is formed into discrete cohesive pellets which are egested into the environment regularly. These pellets are a form of detritus and are important to the storage, transport and transformation of organic matter in a variety of aquatic ecosystems (Wotton and Malmqvist 2001). Faecal pellets are formed from the digested, unassimilated food materials that pass through the invertebrate gut, and during gut passage the food is subjected to breakdown by enzymes and abrasive action as well as an altered pH and in some cases bacterial degradation (Zimmer and Bartholme 2003). Organic matter that is egested as faecal pellets is therefore very different from the materials ingested as food by the invertebrate, having a much altered shape, particle size and chemical composition (Wotton and Malmqvist 2001). As well as unassimilated particulate organic matter (POM), pellets contain a significant amount of dissolved organic matter (DOM) (Jumars *et al.* 1989) and also bacteria which survive gut passage and become localised within the faecal pellet upon egestion (Wotton 1994).

Faecal pellets are common in aquatic ecosystems, both in sediments and suspended in the water column (Wotton and Malmqvist 2001). In the open ocean, zooplankton faecal pellets are abundant in surface waters (Wassmann *et al.* 1999) and much attention has been paid to their role as transporters of organic matter from the photic zone to the ocean floor (Lampitt *et al.* 1990). The faecal pellets of copepods (crustaceans that are highly abundant in the zooplankton) particularly are an important flux of organic matter out of

surface waters of the ocean (Bishop *et al.* 1977, Honjo and Roman 1978, Dunbar and Berger 1981). Copepods are often the most numerous member of the zooplankton in pelagic environments and utilise a wide variety of foods (such as phytoplankton and other zooplankton), the waste then being converted into faecal pellets. They also feed by coprophagy and this has generated much interest due to the implications for nutrient recycling in the photic zone (Lampitt *et al.* 1990, Noji *et al.* 1991). The amount of material copepods assimilate from their food is low (Besiktepe and Dam 2002) and much of the organic matter they ingest remains undigested and egested in their faecal pellets which are bound by a peritrophic membrane secreted from their gut wall ensuring the pellets remain intact when suspended in the water column (Bathelt and Schelske 1983).

Copepods (and other pelagic invertebrates) egest large numbers of faecal pellets that sink through the water column transporting the organic matter they contain with them. As they sink, pellets are subjected to grazing by zooplankton and these processes slow the sinking of the pellets and lead to the retention of the organic matter in the photic zone where it is utilised by invertebrate and microbial communities (Alldredge *et al.* 1987, Lampitt *et al.* 1990, Noji *et al.* 1991). Faecal pellets are especially important as a source of energy to the microbial loop which mineralizes large amounts of the organic matter in sinking faecal pellets and recycles the energy to higher trophic levels (Jumars *et al.* 1989). Pellets that sink out of surface waters become an important part of organic matter aggregates or “marine snow” (Alldredge and Silver 1988, Turner 2002) and transport materials to the ocean floor where they are utilised by the organisms that live on the sea bed (Turner 2002).

Faecal pellets are also important to coastal environments, where they also contribute large amounts of organic matter to coastal sediments (Amon and Herndl 1991). Bivalve molluscs are major contributors of organic matter to sediments through their faecal and pseudofaecal biodeposits (Norkko *et al.* 2001), these sediments then provide a resource for many detritivorous organisms such as polychaete worms which consume the sediment and digest the organic matter it contains, subsequently contributing their own faecal pellets (Wild *et al.* 2005). The production and sedimentation of invertebrate faecal pellets from the water column is also important in supplying organic matter to coastal environments such as seagrass beds (Conde *et al.* 1991) and mangrove swamps (Werry and Lee 2005).

The role of faecal pellets in freshwater ecosystems is much less well understood than the marine environment. Evidence from lakes suggests that faecal pellets there play a similar role in organic matter flux as they do in the ocean, material being mineralized in the upper part of the water column, with only a small proportion reaching the lake bottom (Evans *et al.* 1998). In streams and rivers, faecal pellets can transport huge amounts of material downstream to depositional areas (such as under macrophyte stands or margins of the channel) or to the sea, and the faecal pellets of blackfly larvae (Diptera : Simuliidae) are particularly important to this process (Wotton *et al.* 1998, Malmqvist *et al.* 2001, Warren *et al.* 2005, Wharton *et al.* 2006). Bivalves are also big contributors of faecal material to streams and rivers (Vaughn and Hakenkamp 2001), and the introduction of alien species (such as zebra mussels, *Dreissena polymorpha*) has lead to ecological damage by altering the flux of organic matter within river channels (Strayer *et al.* 1999).

1.3. Lowland Permeable Catchments - Chalk Streams

Chalk streams originate on or flow over lowland permeable chalk geology and this underlying chalk is responsible for many of their characteristics. Chalk streams receive the bulk of their discharge from groundwater sources, namely chalk aquifers over which they flow, and there is little influence of overland flow as the permeable aquifer absorbs much of the rainfall over the catchment. This leads to low drainage density (a relatively low amount of river channel per unit area) and streams that are of low order (relatively few stream branches) (Berrie 1992, Bowes *et al.* 2005). Because most of the discharge of the stream is from groundwater, the source of the stream will move as the water table rises or falls, and more upland parts of many chalk rivers do not flow in summer because of this, as such they are known as “winterbournes” (English Nature & Environment Agency 1999).

Groundwater fed rivers often exhibit a relatively stable discharge regime and resist flash flooding due to the lack of influence of overland flows (Sear *et al.* 1999), and chalk streams are no exception to this (Westlake *et al.* 1970, Bowes *et al.* 2005, Cotton *et al.* 2006). Along with moderated discharge, water temperature is also moderated and changes gradually and predictably over time in chalk streams (Westlake *et al.* 1970, Mackey and Berrie 1991, Berrie 1992). Both these factors produce a relatively stable ecosystem supporting a high biodiversity with exceptional overall biomass of organisms, with a large proportion of this biomass being composed of invertebrate detritivores (Berrie 1992, Pardo and Armitage 1997).

The occurrence of chalk streams is dependent on chalk geology, and as such within the United Kingdom chalk streams occur mainly in chalk hills in Dorset, Buckinghamshire &

Berkshire (the Chilterns), Norfolk, Kent, Lincolnshire and southern Yorkshire, with the highest concentration of streams (which are also the best studied) in Dorset. Chalk streams are listed for priority protection in the UK Biodiversity Action Plan (English Nature and Environment Agency 2004).

The organic component of chalk stream sediments is dominated by invertebrate faecal pellets, with the over 80% of all organic matter in the form of faecal pellets in Dorset chalk streams (Ladle and Griffiths 1980). Particularly abundant are blackfly faecal pellets which are found suspended in the water column and deposited in areas of low flow, especially under macrophyte stands, and this area has been extensively studied under the NERC LOCAR thematic research program with which my programme of study was associated (Warren *et al.* 2005, Cotton *et al.* 2006, Wharton *et al.* 2006). Although the contribution of invertebrate faecal pellets to chalk stream ecology is still not well understood, it is likely that they play an important role in organic matter flux, as a food source for detritivores and as a substrate for the micro-organisms.

1.4. The Freshwater Shrimp *Gammarus* in Chalk Streams

The genus *Gammarus* (Crustacea : Amphipoda) is a common aquatic organism in both marine and brackish freshwater ecosystems throughout the world. It is most well known however in fresh waters where it is common and is found in both European and North American rivers, streams, and lakes. In chalk streams, *Gammarus pulex* is the dominant species and reproduces quickly, reaching 8 – 12 mm in length when adult, and often attains high population densities (Welton 1979). However, *Gammarus* requires high

oxygen concentrations to survive, preferring to live in clean, unpolluted environments often in flowing waters or at the waters edge of lakes and ponds (Rees 1972).

Gammarus is most often described as a shredder by aquatic ecologists, thought to feed mainly on coarse particulate organic matter or CPOM (e.g. dead leaves). Recently however this classification has been questioned as *Gammarus* will feed on a variety of food sources under both laboratory and field conditions, and often cannibalise other *Gammarus* especially when kept at high densities in culture (Dick 1995, MacNeil *et al.* 1997). *Gammarus* even shows a liking for cheese as a food source (Allan and Malmqvist 1989). However, despite their omnivorous nature *Gammarus* clearly play an important role in breaking down allochthonous energy inputs (such as dead leaf fall) into streams as they consume large amounts of dead leaf material that falls into streams and rivers (Kaushik and Hynes 1971).

As with copepods, only a small amount of the material a *Gammarus* ingests is assimilated (Berrie 1976) and it probably gains more nutrition from the micro-organisms growing on the surface of the leaves rather than the leaves themselves (Cummins 1974). The cylindrical faecal pellets that are egested are primarily refractory organic matter and leaf fragments (Ladle and Griffiths 1980) that can be utilised by other organisms in the ecosystem (e.g. in the microbial loop, by coprophagy).

1.5. Ecosystem Engineering and the Ecological Role of Faecal Pellets

Ecosystem engineering can be defined as “a process that directly or indirectly modulates the availability of resources” to an organism, by another organism (Jones *et al.* 1994), and the production of faecal pellets can be seen as a process of ecosystem engineering.

As an organism feeds, it changes the material it is feeding on from one form to another as the faecal pellets it egests are different in size, shape and composition to the original food materials ingested (e.g. a detritivore shredder reduces larger CPOM to *smaller* faecal pellets, a filter feeder collects small organic matter particles from the water column and produces *larger* faecal pellets). Because assimilation efficiency is low, the resulting faecal pellets contain much of the original organic matter and pelletisation alters the fate of the material within the ecosystem, such as how the material is transported and utilised by other organisms (Wotton and Malmqvist 2001). *Gammarus* in chalk streams can be thought of as ecosystem engineers as they convert relatively large dead leaves into smaller faecal pellets which are transported, degraded and utilised differently to the original leaves (e.g. faecal pellets can be consumed by detritivores such as sediment feeders that cannot break down dead leaves, thus they can feed indirectly on allochthonous carbon). When these processes occur on a large scale, such as over a whole stream, large amounts of organic matter can be affected (Wotton *et al.* 1998).

Because the production of faecal pellets occurs on a large scale, its impacts are significant. Pelletisation of sediments enhances retention of organic matter in streams and rivers (Wotton *et al.* 1998, Malmqvist *et al.* 2001, Wharton *et al.* 2006), increases oxygen penetration into marine sediments (Wild *et al.* 2005) and increases the surface area for microbial attachment and degradation (Hargrave 1976, Hansen *et al.* 1996). Because of the abundance of *Gammarus* in chalk streams, it is possible that the production and sedimentation of *Gammarus* faecal pellets may be an ecologically significant process, influencing organic matter flux, storage and utilisation by other chalk stream organisms.

1.6. Aims and Objectives

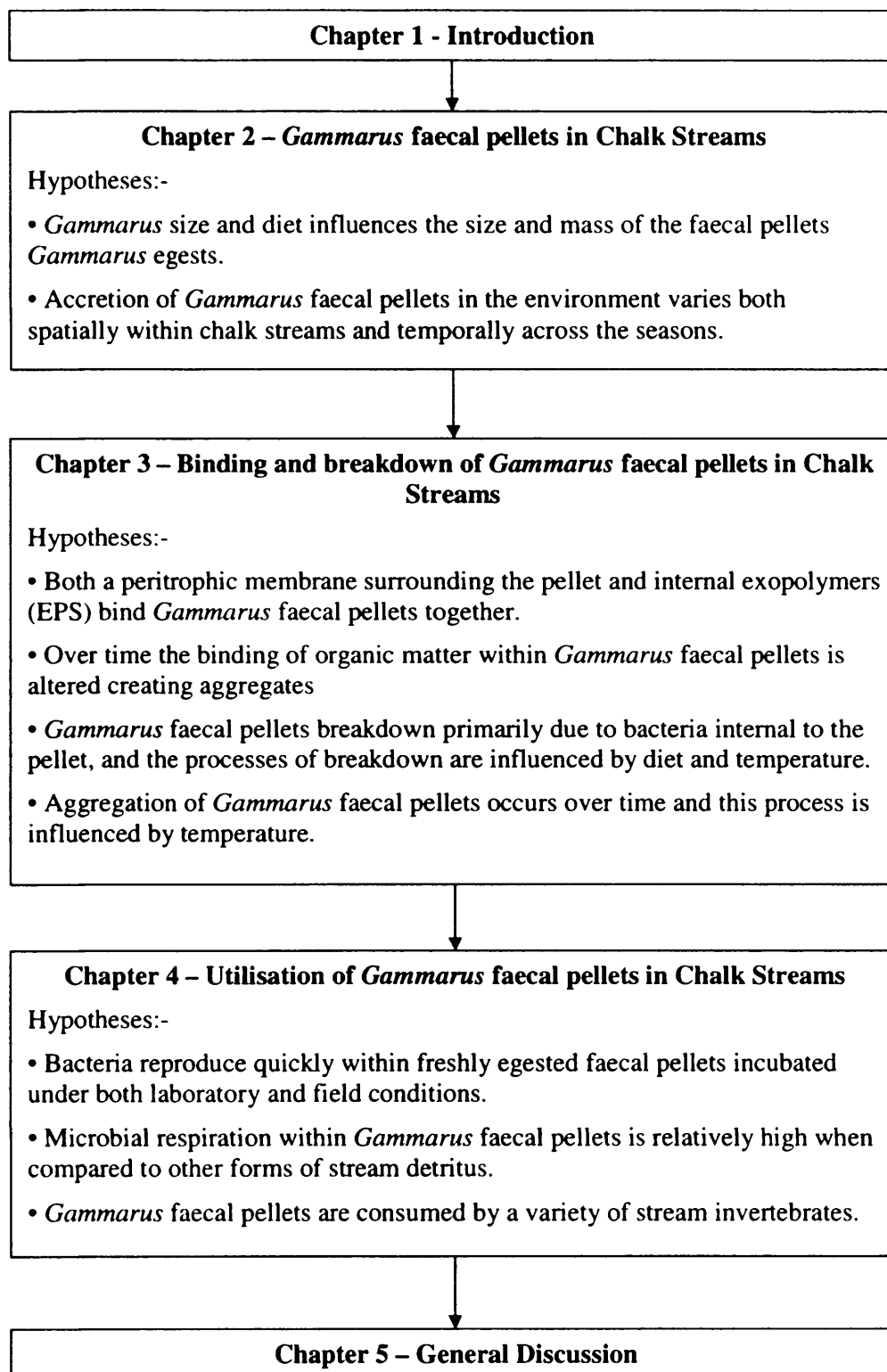
This thesis aims to investigate how *Gammarus* in chalk streams influence the transfer of organic matter by the practice of feeding and producing faecal pellets. This subject is split into three subject areas corresponding to chapters two, three and four of the thesis :

1. *Gammarus* faecal pellets in chalk streams – investigating the physical features of the pellets and their production in the environment.
2. The binding and breakdown of *Gammarus* faecal pellets in chalk streams – investigating the processes that maintain the pellet structure and cause it to break down and release its material back into the environment.
3. Utilisation of *Gammarus* faecal pellets – investigating the fate of the material contained in faecal pellets and its ecological significance to other organisms.

The overall objective is to describe the patterns of occurrence and breakdown of *Gammarus* faecal pellets and determine their significance as an organic matter transfer and storage mechanism in chalk streams.

1.7. Outline and Hypotheses

The following diagram shows the outline structure of each of the three thesis chapters and details the hypotheses investigated in each.



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2. *Gammarus* Faecal Pellets in Chalk Streams

2.1. General Introduction

Faecal pellets, although studied in detail in marine systems (Turner 2002), have been overlooked as transporters of organic materials and as components of benthic organic matter in streams and rivers. The studies that do exist focus mainly on filter feeders as they have a large potential for transforming suspended organic matter into faecal pellets that alter its downstream transport (Wotton and Malmqvist 2001), and most studies have focussed on the faecal pellets produced by larval blackflies (filter feeders that attain very high population densities in flowing waters). Wotton *et al.* (1998) showed that significant amounts of organic matter in the form of larval blackfly faecal pellets, were transported in the current of a Swedish lake-outlet stream 500 metres in length and this material was either deposited on the substratum or lost to the sea. Malmqvist *et al.* (2001) also showed significant transport of material by larval blackfly faecal pellets in large Scandinavian rivers fed by snow melt. In chalk streams, macrophytes growing in the stream channel (especially the water crowfoot *Ranunculus*) trap sediments, including large numbers of blackfly faecal pellets (Wharton *et al.* 2006).

The amphipod *Gammarus pulex* lives in streams with coarse, stony substrata (>1 cm diameter) and which are rich in benthic organic matter such as leaf packs (Gee 1982). It may occur at population densities of over 10,000 individuals m⁻² in chalk streams (Welton 1979) and is one of the most numerous macroinvertebrates found there. As a result, *G. pulex* faecal pellets are common in chalk stream sediments (Ladle and Griffiths 1980). These pellets are cylindrical, range between 0.33 - 1.86 mm in length and 0.17 - 0.43 mm in diameter, and are composed of fine organic detritus with some small identifiable plant fragments (Ladle and Griffiths 1980, Ladle *et al.* 1987). *G. pulex* feeds typically on allochthonous organic matter (such as autumn shed leaves in leaf packs) and the microbial community associated with it (Kaushik and Hynes 1971, Graca *et al.* 1993, Graca *et al.* 1994), and the faecal pellets they egest are a modification of these materials, so that the particle size of the organic matter is greatly reduced (Wotton and Malmqvist 2001). Large amounts of organic matter may be translocated from leaf packs to benthic sediments as *G. pulex* faecal pellets, representing an important flux of allochthonous organic matter for benthic communities in chalk streams.

2.2. The Relationship of *Gammarus pulex* Body Size and Diet to the Size and Mass of Faecal Pellets They Produce

2.2.1. Introduction

The physical characteristics of invertebrate faecal pellets (such as their size, density and composition) affect their role within aquatic environments. In pelagic areas of the ocean (where faecal pellets are produced in large numbers by zooplankton), the rate at which faecal pellets sink is the dominant physical characteristic determining their rate of loss from the photic zone along with the organic matter they contain (Lampitt *et al.* 1990), and size, density and porosity are all characteristics influencing the sinking rate of faecal pellets in the water column (Smayda 1969, Fowler and Small 1972). Faecal pellets produced by pelagic copepods sink faster when large and dense, and these physical characteristics can determine the overall export of organic matter by faecal pellets from the photic zone of the ocean (Small *et al.* 1979, Komar *et al.* 1981, Taghon *et al.* 1984). Porosity of particles also influences sinking rate as a higher porosity leads to increased hydrodynamic resistance, and as a result particles sink more slowly. In flocculated sediment, particle sinking rates are determined mainly by porosity, flocs with higher porosities showing a slower sinking rate (Droppo *et al.* 1997). Transport of faecal pellets in streams and rivers, where the current plays an important role in maintaining particles in suspension, is also influenced heavily by sinking rate. Pellets with a low sinking rate are more likely to be transported in the water column or re-suspended than those with a high sinking rate (Ladle *et al.* 1987).

In benthic habitats (such as underneath macrophyte stands) faecal pellets are deposited directly onto the substratum, or within it where organisms burrow. In these areas flows are low, and as such sinking rates do not affect the transport and deposition of freshly egested faecal pellets from benthic organisms (unless they become re-suspended by the water column). However, physical characteristics of deposited faecal pellets are important as they determine the amount of faecal material entering benthic sediments and how easily that material is re-suspended and lost from the sediments.

Body size and diet both determine the physical characteristics of faecal pellets. The amount of unassimilated nutrients excreted by many aquatic invertebrates in the form of faecal pellets is influenced by their body size, smaller animals excreting a higher proportion of nitrogen, phosphorus and organic matter than larger animals (Vanni 2002, Cross *et al.* 2005, Conroy *et al.* 2005). Body size also correlates with overall faecal pellet size in copepods (Komar *et al.* 1981), bivalves (Giles and Pilditch 2004) and many different chalk stream invertebrates (Ladle and Griffiths 1980).

The effect of changing diet on the physical characteristics of faecal pellets has been studied extensively in marine systems because of the effect it has on particle flux to the ocean floor (Urban-Rich 2001). Changes in the amount of food available to planktonic copepods alters the size (Dagg and Walser 1986, Feinberg and Dam 1998) and rate of production (Honjo and Roman 1978, Butler and Dam 1994, Besiktepe and Dam 2002) of faecal pellets, and in benthic environments diet has profound effects on the quality and transport of horse mussel biodeposits (Miller *et al.* 2002). In freshwater systems, there is also evidence that the diet of zebra mussels influences the transport of their faecal and pseudofaecal deposits (which contain abundant organic matter) (Strayer 1999).

This study focussed on the physical features of *Gammarus pulex* faecal pellets, and tested the hypothesis that *G. pulex* faecal pellet size increases with increasing organism size, and that different diets cause changes in the size of faecal pellets *G. pulex* egests.

2.2.2. Methods – Relationship of Body Size and Diet to Faecal Pellet Size

Experimental Animals

Gammarus pulex were collected from the River Chess in Buckinghamshire and returned to the laboratory where they were held in culture for use in experiments. Plastic aquarium tanks (25 cm length, 15 cm height, 15 cm deep) with a gravel base and aerated with an air pump were used to hold the *G. pulex*. Whilst in culture, they were fed on shed leaves from autumn leaf fall soaked in tap water for 3-4 days. Different species of tree have leaves with different nutritional value in the diet of stream shredders (Webster and Benfield 1986, Irons *et al.* 1988), and protein content is an important determinant of this (Cummins and Klug 1979).

Two tree species, one possessing leaves with high protein content and one possessing leaves with low protein content, were chosen to investigate the effect of diet on faecal pellet size. Both species are commonly found in leaf packs in the River Chess. Leaves were collected from autumn leaf fall and returned to the laboratory and frozen until required. Horse Chestnut (*Aesculus hippocastanum*) leaves were chosen as the high protein / high nutritional value diet and English Oak (*Quercus robur*) leaves were chosen as the low protein / low nutritional value diet. Protein content of the leaves was assessed using the Bradford's dye binding technique and described in Appendix A. Horse Chestnut leaves had a mean protein content of 13.20 mg protein g⁻¹ of leaf mass whereas

Oak leaves had a mean protein content of 7.64 mg protein g⁻¹ of leaf mass. Oak leaves therefore contain half the protein of Horse Chestnut leaves.

G. pulex were fed on these two diets before use in experiments. One group of *G. pulex* were placed in an aquarium tank with only Horse Chestnut leaves as food for 5 days, and one group in an aquarium tank with Oak leaves as food for 5 days. This period allowed the organisms to clear their guts of previously ingested food and fill them with the experimental foods described above.

Apparatus and Procedure

Individual *G. pulex* were placed into small tubes (termed “microcosms”) containing leaf food and left for 3 days to produce faecal pellets. The design of the microcosms is shown in Figure 2.2.1. and consisted of a tube sealed with coarse 1 mm pore size mesh at both ends. The mesh was held in place by elastic bands.

Water was free to circulate through the microcosm but the individual organism was confined. Faecal pellets fell through the mesh and were collected in a 25ml beaker below the microcosm. Replicate microcosms were incubated at room temperature in separate aquarium tanks equipped with air pumps to re-circulate the water throughout the system. To reduce the stress to the organisms, the whole setup was kept in the dark as *G. pulex* actively seek darkened habitats.

Each microcosm was populated with a single *G. pulex* and a 2 cm by 2 cm piece of leaf as food. A total of 45 microcosms were set up and supplied with Horse Chestnut leaves and 45 microcosms were set up and supplied with Oak leaves. *G. pulex* fed on Horse Chestnut leaves were placed in microcosms supplied with Horse Chestnut leaves and this

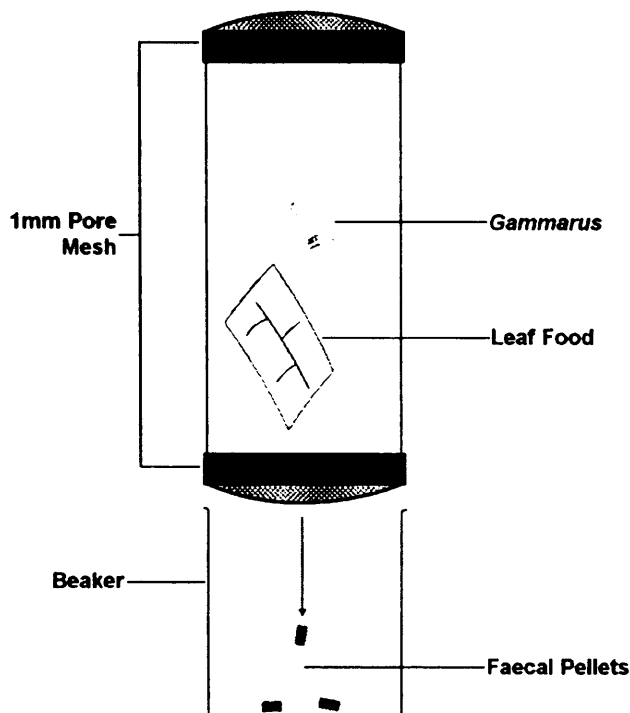


Figure 2.2.1. – Diagram of a single experimental microcosm. Each *G. pulex* was isolated in the tube on top of the beaker with a square of leaf food. Faecal pellets fall through the 1 mm mesh and are collected in the beaker below the tube. Microcosms were submerged in an aquarium tank with a re-circulating air supply.

was similarly done for *G. pulex* fed on Oak leaves. Any microcosms where the *G. pulex* did not produce any faecal pellets were discounted.

After 3 days the tubes were removed. The length from anterior segment of the cephalothorax to posterior tip of the urosome of each *G. pulex* was measured (with the body straightened out of the normally curled position) as this is a previously used indicator of body size (Welton and Clarke 1980). The total number of pellets produced was counted and the diameter of each pellet (*G. pulex* faecal pellets are cylindrical)

produced was measured. Measurements were taken under an Olympus SZ40 binocular microscope with a eyepiece micrometer calibrated against a stage graticule slide.

2.2.3. Methods – Relationship of Body Size to Faecal Pellet Mass

Faecal Pellet Production

A laboratory culture of *Gammarus pulex* was sorted into three body size classes defined by body length. Large *G. pulex* were defined as having 12+ mm body length, medium *G. pulex* 8-11.9 mm body length and small *G. pulex* 1-7.9 mm body length. Approximately 50 individuals of each size class were placed in separate aquarium tanks (25cm length, 15 cm width and 15 cm depth). These tanks contained a ~1 mm mesh net suspended 2 cm above the bottom of the tank. The net allowed faecal pellets to pass through and collect on the base of the tank, but prevented *G. pulex* passing through and feeding on the faecal pellets, disrupting them. *G. pulex* were fed on a variety of autumn-shed leaves collected from the River Chess (a chalk stream in Buckinghamshire and my field study site), a typical diet for them in chalk streams. This method is adapted from one used previously for studies of Arctic amphipods (Werner 2000). Experimental organisms were left to feed for 24-48 hours. After this time the faecal pellets they produced were collected with a pipette for weighing.

Measurement of Faecal Pellet Dry Mass

A total of 10 samples of faecal pellets were collected from each body size class tank, and these pellets were weighed. Before weighing, each pellet sample was digitally photographed, and image analysis (via the freeware package ImageJ) was used to count

the number of faecal pellets in the sample (Appendix B contains a description of the method used for counting and sizing).

The wet faecal pellet samples were placed into 5mm diameter tin foil cups which had been weighed previously and excess water was removed with a pipette. Samples were dried in an oven at 105°C for 24 hours and then weighed on a Sartorius Microbalance (Model SE-2, accurate to 0.1µg) to the nearest µg. The weight of the cup was subtracted from the weight of the cup + dry faecal pellets to give the dry weight of each faecal pellet sample (in mg). A mean weight of individual faecal pellets across the 10 samples taken from each size class was calculated. The total weight of faecal pellets in all 10 samples was divided by the total number of pellets counted by image analysis in all 10 samples. This gave an approximate weight of individual faecal pellets in each size class.

2.2.4. Results – Relationship of Body Size and Diet to Faecal Pellet Size

There was a positive linear relationship between faecal pellet diameter and *G. pulex* body size for both the Horse Chestnut and Oak diets (Figure 2.2.2.). Larger *G. pulex* produce faecal pellets with a larger diameter and smaller *G. pulex* produce faecal pellets with a smaller diameter. As *G. pulex* pellets are on average 3 times as long as their diameter, and are roughly cylindrical, an increase in diameter indicates an increase in volume, and therefore an overall increase in size.

Comparison of the two datasets was carried out in the Minitab 13 statistical analysis software using ANCOVA, with *G. pulex* size as the covariate, showed significant differences ($F = 52.84$ $p < 0.001$ in ANCOVA) between the Horse Chestnut and Oak

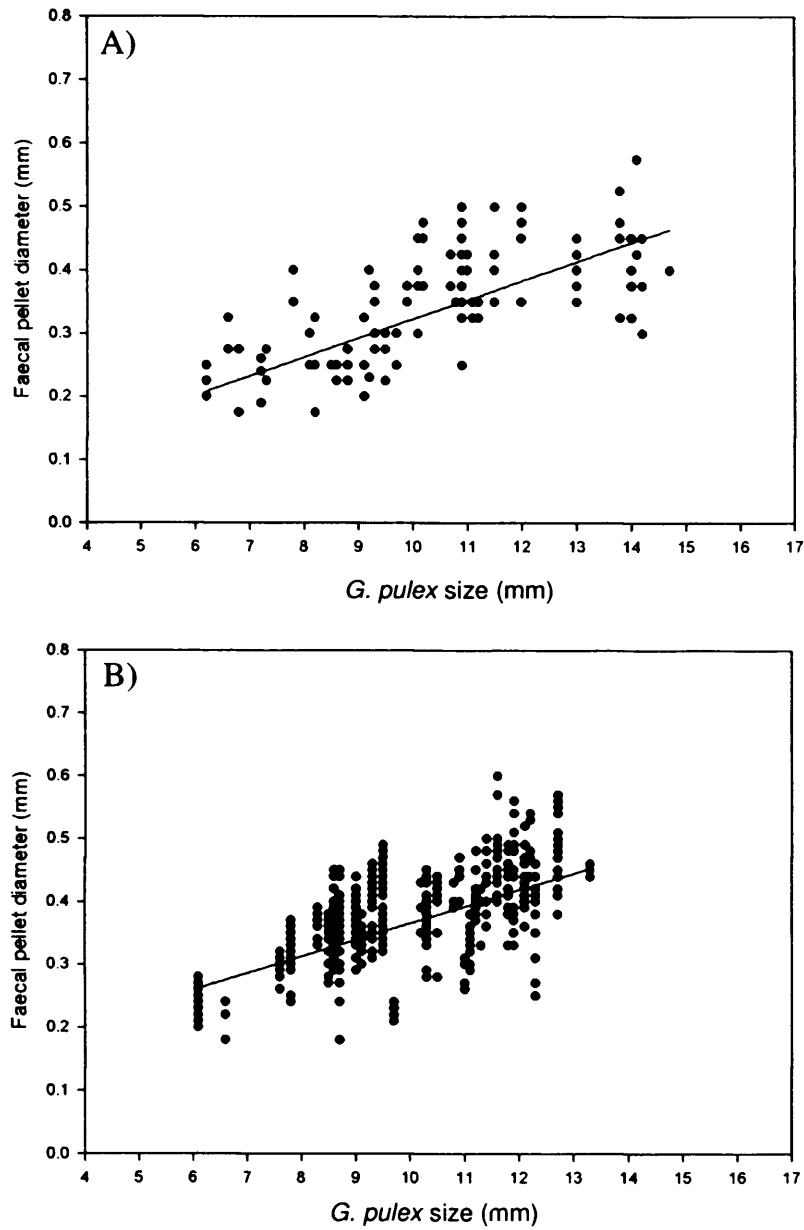


Figure 2.2.2. – Plots and regression lines for *G. pulex* fed on A) Horse Chestnut leaf diet ($y = 0.0213 + 0.0301x$, $r^2 = 0.5535$, $n = 167$, $p < 0.001$) and B) Oak leaf diet ($y = 0.0997 + 0.0266x$, $r^2 = 0.3981$, $n = 510$, $p < 0.001$). Both datasets show a positive linear relationship of *G. pulex* size with faecal pellet diameter.

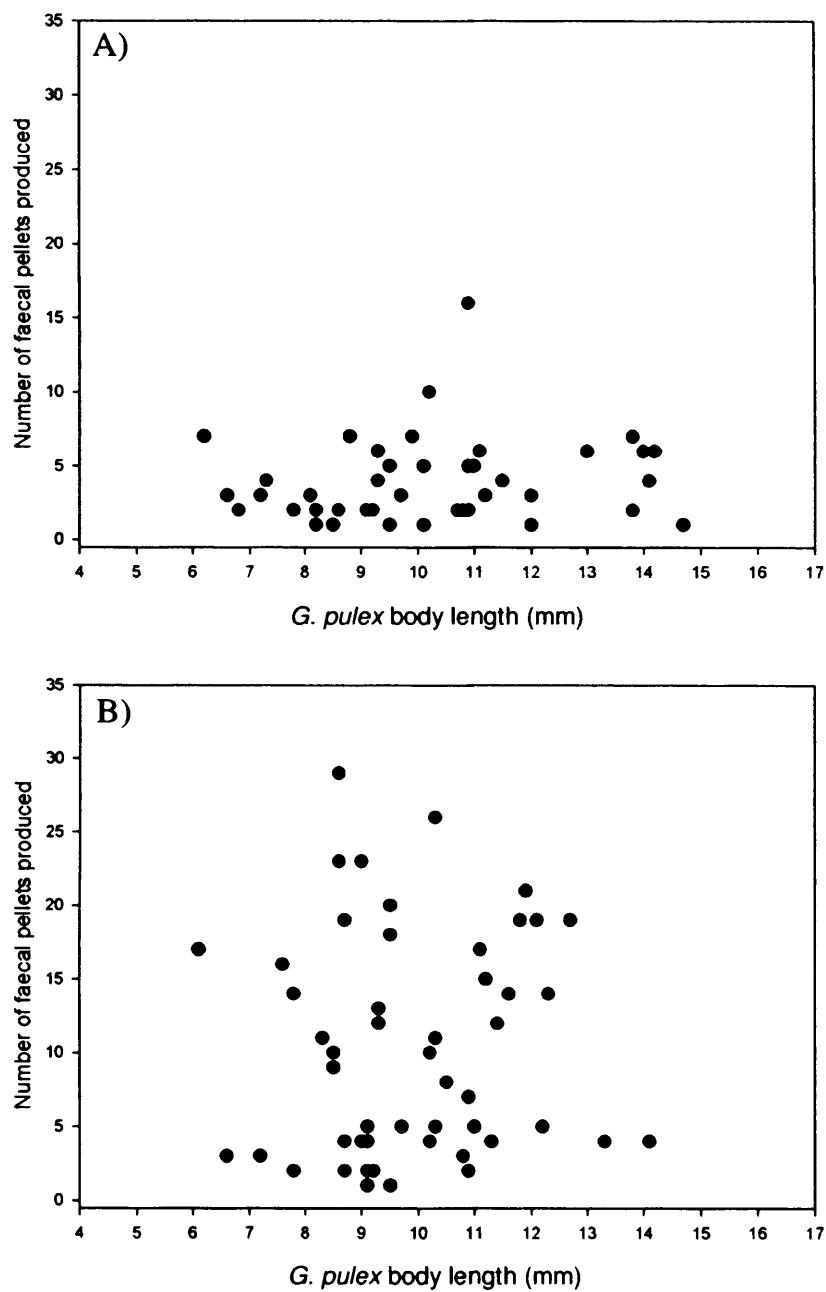


Figure 2.2.3. – Plots showing *G. pulex* body length (mm) against the number of faecal pellets each individual produced when fed on A) Horse Chestnut leaf diet and B) Oak leaf diet. There is no relationship between *G. pulex* body length and the number of faecal pellets it produces for *G. pulex* fed either diet.

datasets. *G. pulex* fed Oak leaves produced significantly larger faecal pellets than those fed Horse Chestnut leaves. Diet also influenced the number of faecal pellets *G. pulex* produced, with *G. pulex* fed Oak leaves producing significantly more faecal pellets than those fed Horse Chestnut leaves ($F = 56.14$ $p < 0.001$ in ANOVA). Therefore, *G. pulex* fed a low quality diet produced more and larger faecal pellets than *G. pulex* fed on a high quality diet. There is no clear relationship between *G. pulex* body size and the number faecal pellets produced (Figure 2.2.3.) for either Horse Chestnut or Oak diets.

2.2.5. Results - Relationship of Body Size to Faecal Pellet Mass

Large body size *G. pulex* (12.0+ mm body length) egested faecal pellets with a mean weight of $25.1 \mu\text{g} \pm \text{SD } 0.0029 \mu\text{g}$, medium body size *G. pulex* (8.0 - 11.9 mm body

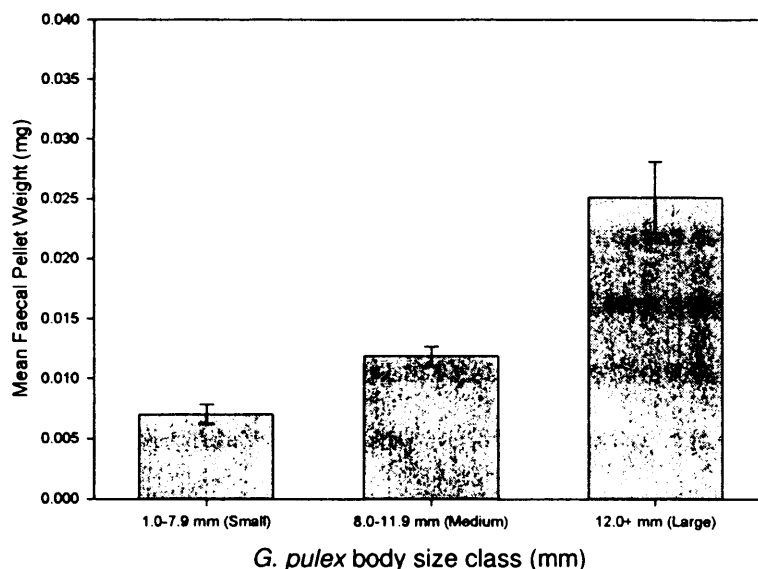


Figure 2.2.4. – Bar chart showing mean faecal pellet weight (mg) for the 3 size classes of *G. pulex* studied (small, medium and large). Bars indicate means and whiskers standard deviations.

length) $11.9 \mu\text{g} \pm \text{SD } 0.0008 \mu\text{g}$ and small body size *G. pulex* (1.0 - 7.9 mm body length) $7.0 \mu\text{g} \pm \text{SD } 0.0007 \mu\text{g}$ (Figure 2.2.3.). The weight of *G. pulex* faecal pellets is related to *G. pulex* body size. Larger *G. pulex* produce faecal pellets with a greater mass.

2.2.6. Discussion

Gammarus pulex body size is an important determinant of faecal pellet size. There was an increase in faecal pellet diameter as body size increased when *G. pulex* were fed both Horse Chestnut and Oak leaves. Ladle and Mathews (1980), reported a similar pattern for several chalk stream invertebrates including *G. pulex*, and Werner (2000) showed that larger body sized *Gammarus wilkitzkii* in the Arctic egest larger faecal pellets.

In the oceans, pelagic copepods with smaller body sizes contribute a greater number of faecal pellets than those with larger body sizes as they have a higher egestion rate and are more numerous (Small and Ellis 1992, Beaumont *et al.* 2001, Cross *et al.* 2005). In freshwaters, *G. pulex* populations contain a greater number of small individuals than large (Welton 1979, Casagrande *et al.* 2006), and therefore, by virtue of numbers, smaller individuals will contribute a greater amount of faecal material than large individuals, despite the fact that large individuals egest more material in each pellet. The body size distribution of an invertebrate population can therefore determine the amount of faecal material the population produces, and therefore the contribution of the organism to organic matter flux in the ecosystem. No evidence for a relationship between body size and number of faecal pellets egested was observed in *G. pulex* here. As such, body size will influence faecal pellet flux in chalk streams because of the larger number of small individuals, rather than by differences in egestion rate.

Faecal pellet mass is also influenced by body size in *G. pulex*. As body size increases, the mass of faecal pellets produced also increases linearly, so that larger individuals produce faecal pellets with a greater mass. There is therefore a link between the size-frequency distribution of the individuals in a *G. pulex* population and the mass of material that population egests as faecal pellets. We would expect most faecal pellets produced by a chalk stream *G. pulex* population to be relatively small because of the higher abundance of smaller *G. pulex* (Welton 1979), and because body size does not determine the rate of faecal pellet production (as shown above in Section 2.2.3.), this relationship will not be attenuated by differences in production rates of different sized *G. pulex*.

The quality of diet available to *G. pulex* significantly alters the size of faecal pellets and the numbers of faecal pellets egested. When fed Oak leaves (the low quality / low protein diet), *G. pulex* produced larger and more numerous faecal pellets than when fed Horse Chestnut leaves (the high quality / high protein diet). Diet therefore can also affect the flux of faecal material from *G. pulex* in chalk streams. When lower quality food items are predominant in the stream, such as winter when most leaves with a high nutritional value have been consumed or broken down (Anderson and Sedell 1979), the size and rate of production of faecal pellets by *G. pulex* is higher. This is because they are forced to feed on lower quality foods (such as Oak leaves) which I have shown to increase the number of faecal pellets egested (see Section 2.2.2.). Studies of other taxa have also shown similarly that diet affects faecal pellet egestion rates in other invertebrates (Giles and Pilditch 2004, Mamelona and Pelletier 2005).

This increase in the rate of faecal pellet egestion when feeding on low quality food has not been observed in studies of pelagic copepods in the oceans. Butler and Dam (1994) found that increasing food quality *increased* the rate of faecal pellet production, not reduced it, and this probably reflects the huge difference in biology between these organisms and *G. pulex*. However, similar to *G. pulex* they found that increasing food quality caused pellets to be smaller. Physical features of faecal pellets are important determinants of the role they play in an ecosystem, and in chalk streams the physical features of *G. pulex* pellets are likely to influence organic matter flux significantly.

2.3. Spatial and Temporal Patterns of Accretion of *Gammarus pulex* Faecal Pellets in Chalk Streams

2.3.1. Introduction

Invertebrate faecal pellets are abundant in many aquatic environments (Wotton and Malmqvist 2001), but their numbers vary both spatially and temporally. Zooplankton faecal pellet abundance in the oceans varies by season (Carroll *et al.* 1998, Riser *et al.* 2002, Dagg *et al.* 2003), by time of day (Edelvang and Austen 1997) and by depth (Wassmann *et al.* 1999). Studies of streams and rivers also show spatial variation in faecal pellet abundance. In unregulated snowmelt rivers in Scandinavia, the concentration of larval blackfly faecal pellets in the water column increases downstream (Wotton *et al.* 1998, Malmqvist *et al.* 2001). Also, in English chalk rivers faecal pellets are more abundant underneath macrophyte stands than in other parts of the river channel (Wharton *et al.* 2006).

Faecal pellets transport and store large amounts of organic matter in chalk streams, with the pellets of larval blackflies (an abundant chalk stream organism) alone releasing $0.87 \text{ kg C m}^{-2} \text{ day}^{-1}$ into the water column, much of which is transported downstream (Warren *et al.* 2005). *Gammarus pulex*, as another abundant chalk stream macroinvertebrate, may contribute large amounts of organic matter as faecal material to the stream, but this is poorly understood. In these experiments I test the hypothesis that the faecal pellets of *G. pulex* are found in some habitats (such as under macrophyte stands) more than others (clear stream channel) in chalk streams, and that faecal pellet abundance in chalk stream sediments varies over time.

2.3.2. Methods – Quantifying *Gammarus pulex* Faecal Pellet Accretion

Using Artificial Substrate Traps to Sample Gammarus Faecal Pellet Accretion

Gammarus pulex is primarily benthic, living in the substratum of rivers, streams and lakes. Different sized *G. pulex* occupy sediments with different sized interstitial spaces (Gee 1982, Pringle 1982, Adams *et al.* 1987). Because *G. pulex* spends most of its time within the substratum, we can expect most of its faecal pellets to accumulate in the interstitial spaces of the stream bed where they are protected from strong currents, and therefore re-suspension. Pellets are likely to be transported (although slowly) by hyporheic flows within the substratum. For this reason we would not expect to find large numbers of *G. pulex* faecal pellets in transport in the water column, as with blackfly faecal pellets which are egested into the current from which the larvae feed (Warren *et al.* 2005). Sampling for *G. pulex* faecal pellets needs therefore to be concentrated on the surface of and within the streambed.

Sediment cores are a widely used method of collecting samples of sediments for analysis and involve driving a strong tube a fixed depth into the substratum and collecting all the material within the tube. In chalk streams, where the stream bed consists of coarse flints and cobbles, coring deep within the substratum where *G. pulex* live and produce faecal pellets is extremely difficult. Artificial substrate traps, where a collecting device is placed within the stream bed and allowed to accumulate material, avoids the problems associated with coring and were therefore used to sample *G. pulex* faecal pellet numbers. The traps used did not allow hyporheic flow to pass through them as the sides of the traps were solid with no holes, and this meant that all the pellets that entered the traps came from the water column, from over the surface of the streambed or from *G.*

pulex within the traps (*G. pulex* could enter the interstitial spaces between the streambed materials in the traps and egest pellets - there was no restriction to *G. pulex* utilising the trap as a habitat). Artificial substrate traps have been used in a variety of studies of benthic macroinvertebrates (Casey and Kendall 1996, Swift *et al.* 1996).

In a field study, artificial substrate traps were used to sample the accretion of *G. pulex* faecal pellets on and within the bed of a chalk stream. Accretion is used here to define the process of accumulation of *G. pulex* faecal pellets on and within the streambed over time, taking into account any inputs from the water column, from movement of pellets over the surface of the streambed, and from *G. pulex* inside the trap egesting pellets. This gave a baseline estimate of the number of *G. pulex* faecal pellets produced within the stream over a fixed period of time. Each trap was filled with streambed materials so that conditions inside the trap replicated those in the streambed of the river as much as possible.

Artificial substrate traps consisted of plastic food containers (14 cm x 14 cm x 7.5 cm) with clip-on lids that were sunk into the substratum so that the top of the container was flush with the stream bed (Figure 2.3.1.). The hole in the streambed into which the trap was placed was lined with wire mesh to stop it collapsing when the trap was removed for collection of samples, and also fixed the trap's location. Traps were cleaned and emptied of all organic sediments and any organisms before the sampling process began. To ensure traps contained no *G. pulex* faecal pellets at the start of the sampling period, they were filled with stream water filtered through a 100 µm sieve to exclude them. A lid was put on the trap as it was placed in the stream, and this prevented pellets entering the trap in

water currents created during positioning of the trap. After a few minutes the water in the stream ran clear of disturbed materials, and the lid was removed to begin sampling.

A sample consisted of the *G. pulex* faecal pellets accreted within a trap over 7 days. Samples were taken by placing a lid on each trap, lifting it out of the mesh-lined hole and collecting the accreted materials. The lid prevented loss of any sample material contained within the trap. The contents of the trap was passed through a coarse sieve (1 mm mesh) in the field to remove any large materials (stones, leaves, organisms, etc...) and the rest of the sample was sealed in a labelled bag with 70% IMS to preserve it. The traps were replaced in the substratum after sampling and were left in place until the next sample was taken to prevent the position of the trap being lost and to make sure all samples taken were from the same location. To prevent any degradation of the material, samples were refrigerated until counted.

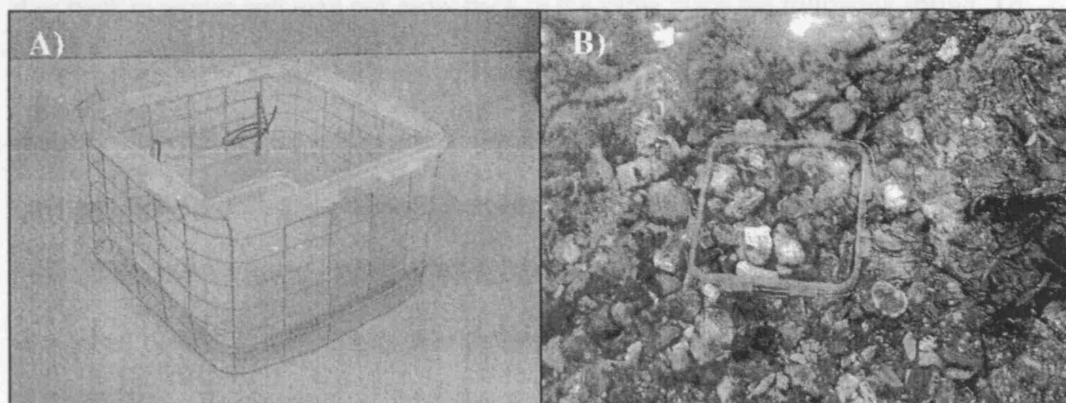


Figure 2.3.1. – Images showing A) a substrate trap and wire frame liner and B) the same trap in the stream bed of the River Chess during sampling.

Location of Field Site

The stream studied was the River Chess, a chalk stream running through Buckinghamshire and Hertfordshire north-west of London (Figure 2.3.2. & 2.3.3.). During 2004 and 2005, when field work was carried out, mean annual discharge of the river (measured at Rickmansworth monitoring station) was 0.395 and 0.239 m³ sec⁻¹ respectively (National River Flows Archive - <http://www.nwl.ac.uk/ih/nrfa/index.htm>). A small reach near the village of Latimer was chosen for the study as it provided easy access.

Spatial Patterns of Faecal Pellet Accretion

Vegetation cover over the substratum of chalk streams changes throughout the year, and it is not possible to sample the same patch of vegetation for a long period of time as it dies back in winter and may not grow back in the same place the following spring. To sample accretion of *G. pulex* faecal pellets in areas of different habitat types, 3 line transects were set up across the stream channel and substrate traps were placed at regular intervals along these transects. Each transect consisted of 8 traps, giving a total of 24 traps across the 3 transects (Figure 2.3.3.). Traps were sampled at different time periods (described below) so that accretion of pellets could be measured for all types of vegetation cover within the stream, as vegetation grew over the traps during the spring and summer, and died back over the autumn and winter.

Vegetation type within the stream was classified into three categories, similar to those used by Welton (1980): 1) Marginal vegetation (consisting mainly of *Nasturtium*, but also other aquatic macrophytes, 2) *Ranunculus* beds mainly in the middle of the stream,

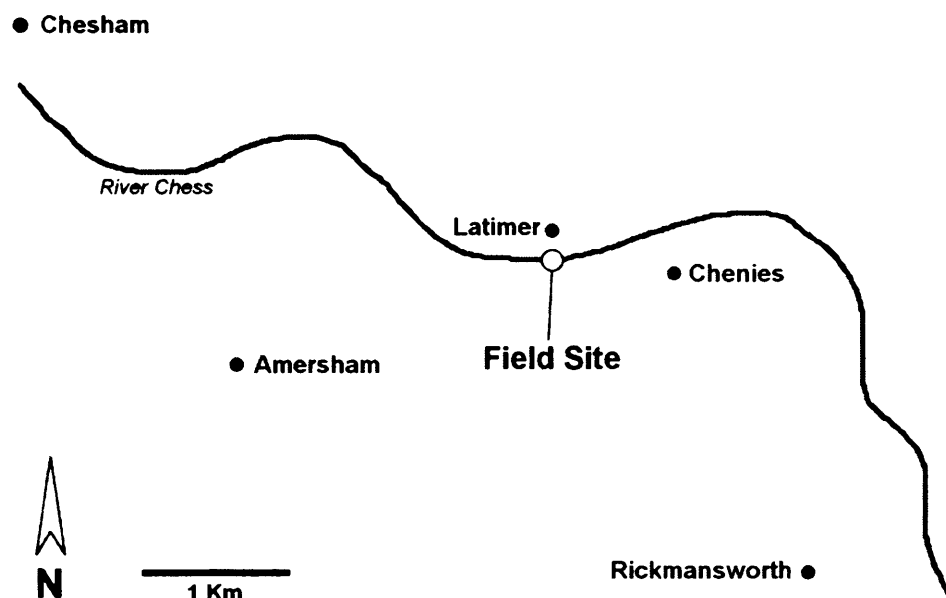


Figure 2.3.2. – Map showing the location of the field site used for sampling.

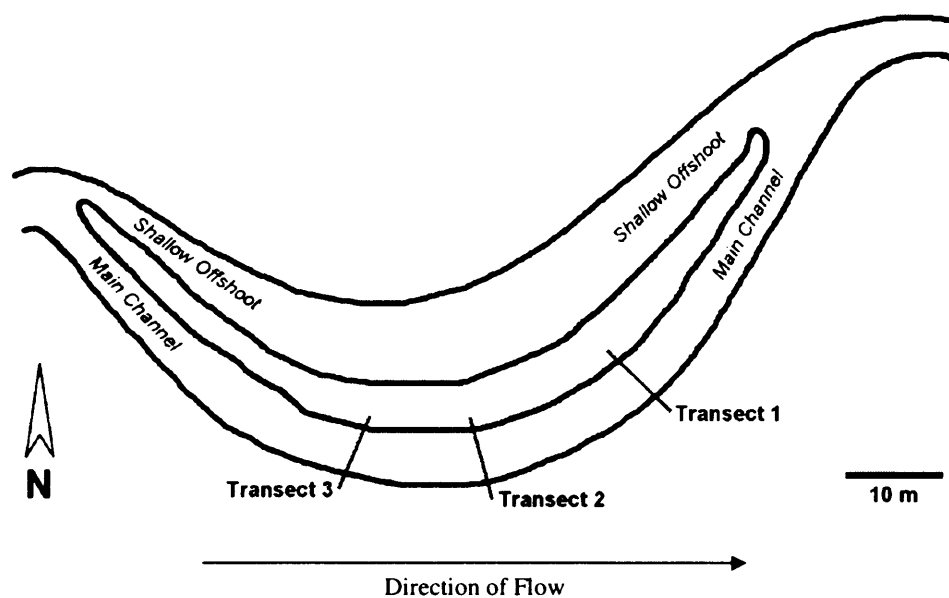


Figure 2.3.3. – Diagram of the stream reach used and the location of the 3 transects within the reach.

and 3) clear channel with no vegetation.

Temporal Patterns of Faecal Pellet Accretion

Samples of the numbers of *G. pulex* faecal pellets accreted in traps over one week were taken every 3 months, the first sample in December 2004, the second March 2005, the third June 2005 and the last September 2005. By comparing accretion during different times of the year, it was possible to see if there were any times when accretion was high and low.

Counting Gammarus pulex Faecal Pellets Captured in Substrate Traps

To quantify the numbers of faecal pellets within each sample, volumetric sub-sampling was used, similar to that by Wotton *et al.* (1998). Each sample was passed through a 50 µm pore-size sieve with excess tap water to remove the 70% IMS. The material was then washed from the sieve into a bottle, which was then filled with distilled water to a total volume of 500 ml.

To count the number of pellets in the sample, the bottle was shaken vigorously to suspend the particles and a 1 ml sub-sample taken quickly using a pipette. This did not cause disruption of faecal pellets. The sample was placed into a Sedgewick-Rafter cell (1 ml capacity), and the number of *G. pulex* faecal pellets counted under a binocular microscope. The sub-sample was returned to the bottle and the process repeated for 20 sub-samples. Using laboratory produced faecal pellets, this method was tested for its accuracy and gave a coefficient of variation of 30.3%. Although this is higher than that

calculated in Wotton (1998), it was deemed to be sufficiently low for the method to give valid results.

For each sample, a mean of the sub-sample counts was taken, and then multiplied by 500 (the total volume of the suspended sample) to give an estimate of the number of faecal pellets in each sample. Where there were large numbers of particles in the sample (i.e. when faecal pellets overlapped and their numbers could not be counted), samples were diluted 2 – 4 times before counting and the calculation of the number of pellets adjusted by the dilution factor accordingly.

G. pulex faecal pellets were easy to distinguish from those of other organisms. They were identified using the descriptions in Ladle and Griffiths (1980) and photomicrographs of pellets harvested from laboratory cultures of *G. pulex*. The only faecal pellets in chalk streams that may be confused with *G. pulex* pellets are those of *Asellus*, but these are easily separated as *Asellus* pellets have a longitudinal groove running along the pellet surface (Ladle and Griffiths 1980).

Data Analysis

The surface area of substrate traps open to capture faecal pellets and to colonising *Gammarus* was 175 cm² and the number of pellets accreted over 7 days were expressed per m². For each transect, one-way ANOVA was used to look for differences within the data. Tests were carried out to look for differences between habitat types (marginal vegetation, *Ranunculus* and clear channel) and periods when samples were taken (December 2004, March 2005, June 2005, September 2005).

2.3.3. Methods – Estimation of the Mass of *Gammarus pulex* Faecal Pellets Accreted

The maximum and minimum mass ($\text{g m}^{-2} \text{ day}^{-1}$ dry mass) of *Gammarus pulex* faecal pellets accreted in the River Chess was estimated using data collected from the artificial substrate traps. Chalk stream *G. pulex* populations contain individuals of a variety of sizes, with body lengths ranging from ~1 mm to ~13 mm (Welton 1979). As shown in Section 2.2.4., faecal pellet size and mass are related to *G. pulex* body size, and consequently the sizes of faecal pellets collected in samples from the stream will reflect the range of body sizes of the *G. pulex* population there.

To estimate the amount of organic matter present as *G. pulex* faecal pellets in the River Chess, the counts of faecal pellets accreted in substrate traps were converted into estimates of accreted mass of material. These estimates were based on measurement of faecal pellet mass from Section 2.2.5. To account for the influence of the variety of body sizes of the *G. pulex* population, the range of possible accreted mass was determined, the actual value is within these limits. A maximum accreted mass was calculated by assuming every pellet was produced by a “large” (12+ mm body length) *G. pulex* and a minimum accreted mass by assuming the opposite, that every pellet was produced by a “small” *G. pulex* (1-7.9 mm body length), the same size categories defined in Section 2.2. The count of pellets from each sample was multiplied by 0.0251 (the mean mass in mg of faecal pellets from large *G. pulex*) to give the maximum possible accreted mass, and 0.0070 (the mean mass in mg of faecal pellets from small *G. pulex*) to give the minimum possible accreted mass. This maximum / minimum estimate of accreted mass allowed comparison with literature data sets describing organic matter flux in other aquatic systems.

2.3.4. Results - Quantifying *Gammarus pulex* Faecal Pellet Accretion

Spatial Patterns of Faecal Pellet Accretion

The vegetation cover of traps did not influence the rate of *G. pulex* faecal pellet accretion in chalk stream sediments (Figure 2.3.4.). There was no significant difference in pellet accretion rate between the three vegetation types in transect 1 ($F_{2,31} = 3.141$ $p = 0.0582$ in one-way ANOVA), transect 2 ($F_{2,31} = 1.418$ $p = 0.258$ in one-way ANOVA) or transect 3 ($F_{2,31} = 2.007$ $p = 0.152$ in one-way ANOVA). *G. pulex* faecal pellets are therefore not found in higher numbers underneath macrophyte stands (either marginal vegetation or *Ranunculus* beds in mid channel) than in areas with no vegetation.

Temporal Patterns of Faecal Pellet Accretion

There was seasonal variation in the rate of accretion of *G. pulex* faecal pellets (Fig. 3.). When tested with ANOVA, transect 1 ($F_{3,31} = 14.79$ $p < 0.001$ in one-way ANOVA), transect 2 ($F_{3,31} = 14.79$ $p < 0.001$ in one-way ANOVA) and transect 3 ($F_{3,31} = 14.79$ $p < 0.001$ in one-way ANOVA) all showed that there were significant differences between sampling periods.

When analysed with *post-hoc* tests, all three transects showed a similar pattern of differences (Table 1.). Accretion rates of *G. pulex* faecal pellets did not differ significantly in December 2004, March 2005 and June 2005 (all $p < 0.001$ in Tukey-Kramer *post-hoc* test), but accretion rate of these three sampling periods differed significantly from that observed in September 2005 (all $p > 0.05$ in Tukey-Kramer *post-hoc* test). The rate of *G. pulex* faecal pellet accretion in chalk streams is therefore significantly higher (by approximately 10-20 times) than in autumn.

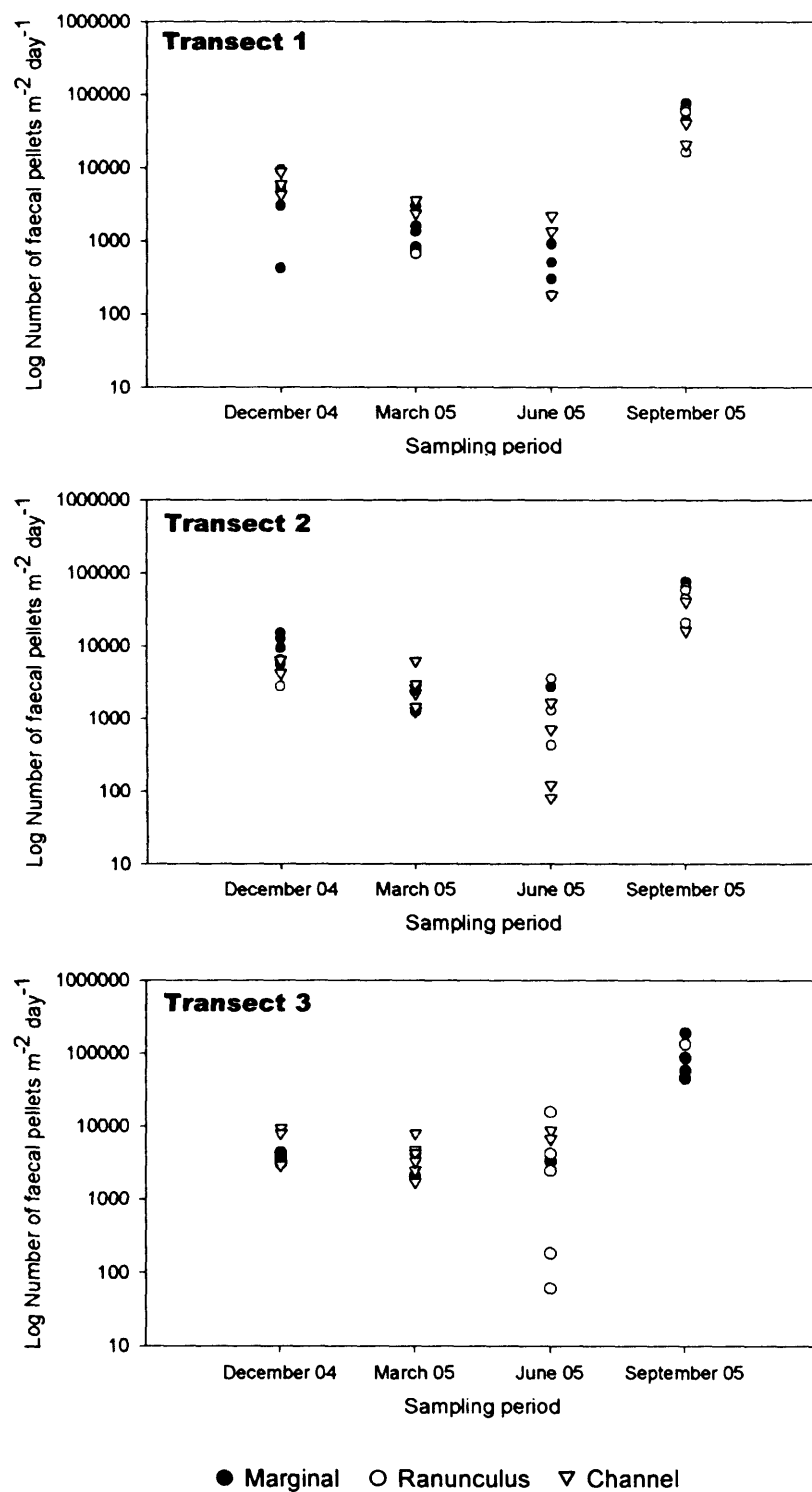


Figure 2.3.4. – *G. pulex* faecal pellets deposited in artificial substrate traps across 3 transects in the River Chess, sampled on 4 occasions.

2.3.5. Results – Estimation of the Mass *Gammarus pulex* Faecal Pellets Accreted

The maximum and minimum mass of *Gammarus pulex* faecal material accreted across the three transects changed substantially over time (Table 2.3.1.). The range of mass accreted in December 2004 was 3 – 382 mg m⁻² day⁻¹, 4 – 201 mg m⁻² day⁻¹ in March 2005, 0.4 – 392 mg m⁻² day⁻¹ in June 2005 and 107 – 5292 mg m⁻² day⁻¹ in September 2005. The true mass accreted lies within these ranges. Several traps showed no accretion of pellets, and in all cases this was because, during sampling, that part of the stream channel was not submerged, and data from these traps has therefore been omitted.

The greatest amount of material was accreted at the beginning of Autumn in September 2005. In this month the mean mass of *G. pulex* faecal pellets accreted in the River Chess (given as the mass \pm SD) was 442.8 \pm 372 – 1587.8 \pm 1333 mg m⁻² day⁻¹. This was approximately 20 times higher than the sampling period in spring (March 2005) and summer (June 2005). In March 2005 mean accreted mass was 18.9 \pm 12 – 67.3 \pm 43 mg m⁻² day⁻¹, and June 2005 was 16.8 \pm 25 – 60.5 \pm 92 mg m⁻² day⁻¹. Accretion in September 2005 was 10 times higher than in the winter sampling period (December 2004) where mean accreted mass was 43 \pm 24 – 155 \pm 88 mg m⁻² day⁻¹. Autumn and winter are therefore important times of accretion of *G. pulex* faecal pellets, whereas in summer months few are accreted. Traps did not record mass accreted due to hyporheic flows as these were excluded by the sides of the trap, and as such, these are conservative estimates of accreted mass.

A) Transect 1 – minimum / maximum mass $\text{mg m}^{-2} \text{ day}^{-1}$

	Trap 1	Trap 2	Trap 3	Trap 4	Trap 5	Trap 6	Trap 7	Trap 8
December 2004	3.0 / 10.8	21.4 / 76.8	61.7 / 221.3	42.1 / 151.1	30.1 / 108.1	66.3 / 237.7	38.0 / 136.3	64.9 / 232.6
March 2005	5.9 / 21.0	21.1 / 75.8	16.9 / 60.4	4.7 / 16.9	5.4 / 19.5	9.4 / 33.8	11.3 / 40.5	25.0 / 89.6
June 2005	1.3 / 4.6	0.0 / 0.0	15.6 / 55.8	9.6 / 34.3	1.3 / 4.6	2.1 / 7.7	6.4 / 23.1	3.6 / 12.8
September 2005	159.1 / 570.6	354.6 / 1271.4	578.4 / 2074.1	361.6 / 1296.5	107.7 / 386.2	836.1 / 2998.2	1223.4 / 4386.9	546.9 / 1960.9

B) Transect 2 - minimum / maximum mass $\text{mg m}^{-2} \text{ day}^{-1}$

	Trap 1	Trap 2	Trap 3	Trap 4	Trap 5	Trap 6	Trap 7	Trap 8
December 2004	45.6 / 163.4	106.9 / 383.2	29.6 / 106.0	19.6 / 70.2	66.6 / 238.7	39.4 / 141.4	89.0 / 319.1	45.3 / 162.4
March 2005	15.4 / 55.3	18.4 / 66.1	16.4 / 58.9	8.9 / 31.8	20.9 / 74.8	8.9 / 31.8	10.0 / 35.9	43.6 / 156.2
June 2005	19.1 / 68.6	25.0 / 89.6	3.0 / 10.8	9.1 / 32.8	0.9 / 3.1	0.6 / 2.0	5.0 / 17.9	11.6 / 41.5
September 2005	0.0 / 0.0	534.9 / 1917.8	310.6 / 1113.6	146.3 / 524.5	450.6 / 1615.6	410.9 / 1473.2	120.9 / 433.4	416.9 / 1494.7

C) Transect 3 - minimum / maximum mass $\text{mg m}^{-2} \text{ day}^{-1}$

	Trap 1	Trap 2	Trap 3	Trap 4	Trap 5	Trap 6	Trap 7	Trap 8
December 2004	21.3 / 76.3	26.9 / 96.3	65.0 / 233.1	56.0 / 200.8	20.4 / 73.3	30.4 / 109.1	25.6 / 91.7	22.7 / 81.4
March 2005	14.4 / 51.7	33.3 / 119.4	56.1 / 201.3	12.0 / 43.0	17.4 / 62.5	26.7 / 95.8	29.9 / 107.1	23.1 / 83.0
June 2005	23.3 / 83.5	0.4 / 1.5	29.6 / 106.0	109.4 / 392.4	1.3 / 4.6	17.3 / 62.0	62.3 / 223.3	47.3 / 169.6
September 2005	0.0 / 0.0	315.7 / 1132.1	324.9 / 1164.8	406.9 / 1458.9	934.3 / 3350.1	611.1 / 2191.4	1476.0 / 5292.5	0.0 / 0.0

Table 2.3.1. – Minimum and maximum mass of *G. pulex* faecal material ($\text{mg m}^{-2} \text{ day}^{-1}$) deposited across 3 artificial substrate trap transects in the River Chess over a 12 month period.

2.3.6. Discussion

Gammarus pulex faecal pellet accretion is not affected by vegetation type, and does not show spatial variation in the River Chess, contrary to my hypothesis that pellets would be found in larger numbers under macrophyte stands than in areas of clear channel with no vegetation. This differs from organic matter accretion on the streambed of chalk streams which shows a distinct spatial pattern, with large amounts being accreted at the margins and under mid-channel *Ranunculus* stands, and with little accreted in areas of clear streambed (Cotton *et al.* 2006, Wharton *et al.* 2006). These areas of accretion have slow flows which cause sedimentation of transported detritus, including large numbers of larval blackfly faecal pellets which can attain standing crops of up to 2.2×10^8 per m² (Wharton *et al.* 2006). The growth of vegetation in chalk streams, especially of *Ranunculus* plants, determines the spatial pattern of organic matter accretion within the stream channel (Cotton *et al.* 2006, Wharton 2006). *G. pulex* faecal pellets do not show a similar pattern of accretion and are accreted in similar numbers under marginal macrophytes, *Ranunculus* stands and in clear stream channel.

This probably reflects the biology of *G. pulex* which is primarily a benthic detritivore, spending most of its time in chalk streams between the coarse cobbles and flint of the substratum. Consequently, *G. pulex* faecal pellets are accreted mostly within the substratum. Pellets that may be eggested when the organism is elsewhere in the stream, such as swimming in the water column, quickly sink and are accreted without being transported far due to their high sinking rate (Ladle *et al.* 1987). This benthic character of *G. pulex* faecal pellets sets them apart from other faecal pellets which are easily transported, such as those of *Simulium* spp. larvae. These pellets are released into the

water column in fast-flowing parts of the stream channel which form the feeding current for the larvae (Wotton 1992). They are then trapped en-mass within stands of *Ranunculus* (Wharton *et al.* 2006).

Because *G. pulex* faecal pellets are egested within the substratum away from the highest current velocities of the stream (although they will be subjected to hyporheic flows), re-suspension and transportation in the water column will be rare. Therefore, they will not be trapped by stands of vegetation such as *Ranunculus* as *Simulium* faecal pellets are. This, combined with the high mobility of *G. pulex* within the stream channel, so that they are not localised within a single area (Allan and Malmqvist 1989, Williams and Williams 1993), probably causes the lack of a spatial pattern in the accretion of their faecal pellets.

Although no spatial pattern was found in the accretion of *G. pulex* faecal pellets, a strong temporal pattern was observed supporting my hypothesis that this would be the case. Pellet accretion decreased from December 2004 to June 2005, and then peaked in September 2005. A combination of two factors are most likely to control this pattern – the population of *G. pulex* and the availability of allochthonous materials as food.

G. pulex populations in Dorset chalk streams such as the Tadnoll Brook reach their highest densities in September and lowest densities in February (Welton 1979). Populations peak in summer, remain high through September into autumn and decline to a low in winter. Other *in-situ* studies on *Gammarus* have shown this pattern of abundance is a common feature of *Gammarus* populations (Graca *et al.* 1994). *Gammarus pseudolimnaeus* in temperate streams in the USA were highest in August at ~6000 individuals m⁻² and lowest in January / February at ~125 individuals m⁻² (Waters and Hokenstrom 1980), *Gammarus aequicauda* in a Mediterranean brackish lagoon peaked

later in September/October at 1800 individuals m⁻² (Casagrande *et al.* 2006), and the pelagic amphipod *Hyaella montezuma* (similar to *Gammarus*), showed a large peak in population in summer (July/August) even though it is known to be a multivoltine species breeding around 3 times a year (Dehdashti and Blinn 1991). It is likely that *G. pulex* populations in the River Chess follow a similar pattern, with a peak of abundance in late summer and the lowest abundance in winter.

The highest numbers of faecal pellets were found in mid to late September when *G. pulex* populations are likely to be at their highest numbers. September is also the start of Autumn leaf fall and many leaf packs were already present in the River Chess when the September sample was taken. The peak in faecal pellet accretion seen in September 2005 therefore coincides with a major seasonal input of allochthonous organic matter, a primary food source of shredders such as *G. pulex*. Allochthonous materials are quickly utilised by *G. pulex* after a short period of microbial conditioning (Kaushik and Hynes 1971), leading to the production of large amounts of faecal pellets which accrete on and in the substratum.

The March 2005 sample follows the opposite pattern. *G. pulex* populations are at their lowest in winter and early spring and at this time few allochthonous materials are left within the stream due to feeding pressure and washing out of materials by high winter flows in the stream. Numbers of faecal pellets being produced and accreted by *G. pulex* are therefore low. This trend is continued into the early summer as shown by the low numbers of pellets accreted in traps in June 2005.

Populations of *G. pulex* in Dorset chalk streams consist mainly of small individuals (~85%) with some large adults (Welton 1979, Adams *et al.* 1987). Amphipod populations

in other environments also show a similar predominance of smaller individuals (Dehdashti and Blinn 1991, Casagrande *et al.* 2006). As such, amounts of *G. pulex* faecal material that accrete in the stream are likely to be closer to my minimum rather than maximum estimates.

My estimates of the maximum and minimum mass of *G. pulex* faecal material accreted in the River Chess are mostly in the low mg quantities, only in September 2005 did estimates increase above $1 \text{ g m}^{-2} \text{ day}^{-1}$. Welton (1980) showed that total sediment accretion (in a 70 mm diameter / 0.15 m^2 surface area sediment trap) ranged between 10 and 270 g dry weight week^{-1} , a significant proportion being organic matter, and clearly much higher than even the maximum estimated mass of *G. pulex* faecal pellets accreted in the River Chess. Only in September 2005 could it approach a significant proportion of the total organic matter accreted in sediments. The mass of organic matter accreted as *G. pulex* faecal material in the River Chess therefore probably does not make up a large fraction of the total benthic organic matter on and in the substratum most of the year. Ladle and Griffiths (1980) agree with this hypothesis, placing *G. pulex* faecal pellets below those of oligochaetes and *Simulium* in order of abundance in chalk stream sediments.

G. pulex faecal pellets may store allochthonous carbon on and in the streambed, which can be utilised by a variety of organisms within the stream, as well as *G. pulex* itself. This is expanded later in Section 4.2. and 4.3. where the processes that utilise faecal pellets within the stream are discussed.

2.4 General Discussion

Gammarus pulex faecal pellets in chalk streams show no spatial pattern of abundance and can be found in large numbers within the substratum of areas without vegetation, under marginal macrophyte stands and under *Ranunculus* beds. This differs from the highly spatial pattern of deposition seen in blackfly faecal pellets which are deposited in larger numbers under *Ranunculus* beds than areas with no vegetation (Warren *et al.* 2005, Wharton *et al.* 2006). *G. pulex* faecal pellets are not transported and deposited in a similar way to *Simulium* faecal pellets. The distribution of *G. pulex* pellets within chalk streams such as the Chess therefore depends mainly on the behaviour of the organism and not characteristics of water flow within the stream channel, as would be expected from an organism living largely within the streambed.

Although lots of evidence exists documenting the preference of *G. pulex* for areas of streambed free from fine sediments (such as silt) and with large pore-spaces (Gee 1982, Pringle 1982), most studies did not take into account changes in behaviour that occur at night. *G. pulex* is most active at night when it leaves the relative safety of the substratum (Allan and Malmqvist 1989) and large amounts of upstream/downstream migration occurs (Hultin 1971, Iversen and Jessen 1977). Its behaviour changes, and individuals move all over the stream, utilising different food resources and habitat types, and the situation is further complicated as individuals of different age have different habitat preferences (Elliott 2005). These patterns of behaviour, far less predictable than those of a sessile organism such as *Simulium*, allow *G. pulex* to access a wide variety of habitats across the stream channel, and probably determines the lack of spatial variation in faecal pellet abundance. Because *G. pulex* effectively range over the whole stream, they will

deposit pellets over the whole stream, and as their pellets are not easily transported they will not be trapped by vegetation and accumulate under macrophyte stands.

The flux of material deposited as *G. pulex* faecal pellets is influenced by the quality of the foods available to them, and by the size-frequency distribution of the *G. pulex* population. Diet influences both the size and rate of production of *G. pulex* faecal pellets, and body size also influences the size of faecal pellets egested (as shown in Section 2.2.). The availability of food resources and the population dynamics of *G. pulex* therefore influence the abundance of faecal pellets in the environment (as discussed in Section 2.3.). As both of these factors change with time in chalk streams, variation in faecal pellet abundance is expected. Counts of the numbers of faecal pellets deposited in the River Chess over time (Section 2.3.) show this as numbers were low in March and June 2005 (spring and summer) and increased to a peak in September 2005 (autumn). This autumn peak (where mean estimates of minimum and maximum accretion were 0.44 and 1.58 g m² day⁻¹ respectively) was not repeated on any other sampling periods and accretion of *G. pulex* faecal material during the rest of the year was 10-20 times lower. *G. pulex* faecal pellets therefore will be most important to organic matter flux in autumn when large amounts of allochthonous materials (from leaf fall) will be present in the stream.

G. pulex is thought of primarily as a shredder that feeds on allochthonous materials, and their feeding on leaf fall in autumn probably explains the increase in deposition at this time. It is not entirely clear however what *G. pulex* feeds on in the summer when these materials are not available, although fine detritus particles and their associated microbial community have been suggested (Willoughby and Earnshaw 1982), along with autochthonous production such as diatoms and algae (Gayte and Fontvieille 1997).

Emergent vegetation does not feature in the diet of *G. pulex* because of well developed biochemical feeding deterrent in the physiology of chalk stream plants (Newman *et al.* 1992).

G. pulex breeds primarily in the summer months (Embody 1911, Iversen and Jessen 1977, Welton 1979) when there is little or no remaining material from autumn leaf fall in the stream, and clearly there must be other sources of nutrition which the population can utilise. Adult *G. pulex* are forced to feed on other sources of detritus, and coprophagy may become an important source of nutrition during these times. Juvenile *G. pulex* are also known to be coprophagous on adult faecal pellets (McCahon and Pascoe 1988), a process also seen in aquatic Isopods (Costantini and Rossi 1998). Coprophagy causes disruption of pellets (Noji *et al.* 1991), and this may account for the low numbers seen in the March 2005 and June 2005, as during these months *G. pulex* may have been feeding by coprophagy and lowering the abundance of pellets in the River Chess. If coprophagy causes the large-scale disruption of *G. pulex* faecal pellets then my data describing the accretion of pellets in the River Chess is a conservative estimate. This is because only intact pellets were counted, and counts did not include pellets that were partially degraded or that had broken down (releasing their materials).

The faecal pellets egested by *G. pulex* are an important input of organic matter to benthic sediments in chalk streams in autumn, when leaf fall and its associated organic matter is available to them as a food resource. This organic matter is distributed throughout the stream and does not become localised under macrophyte stands (either marginal or in-stream *Ranunculus* beds). During the rest of the year faecal pellet numbers in benthic sediments fall, and this may be due to coprophagy by *G. pulex* and other chalk

stream organisms utilising the pellets as food. Breakdown processes, such as those brought about by micro-organisms may also be responsible for the decrease in pellet numbers in spring and summer, and may change the organic matter within faecal pellets and its availability to other organisms within the stream. These subjects are explored in the next chapter, where the binding and breakdown of faecal pellets is investigated.

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3. The Binding and Breakdown of *Gammarus pulex* Faecal Pellets in Chalk Streams

3.1. General Introduction

The faecal pellets of aquatic invertebrates are made up of organic matter (mostly undigested food) bound into a matrix (Wotton and Malmqvist 2001), possibly with some mineral particles also present (Ladle and Griffiths 1980). Binding materials maintain the shape and structure of faecal pellets, and without these pellets would fall apart. During the breakdown of faecal pellets, binding materials are lost and many characteristics of the pellet are altered (such as shape and size), and when the binding of the pellet breaks down, particles are released from the matrix into the environment. Dissolved organic matter (DOM) is also present and is leached from pellets soon after egestion (Jumars *et al.* 1989).

The breakdown of marine copepod faecal pellets both by physical disruption and microbial breakdown has received much attention, as the large-scale breakdown of pellets influences organic matter flux in the photic zone of oceans (Hansen *et al.* 1996). Physical disruption has been shown to reduce the sinking rate of copepod faecal pellets and leads to retention of organic matter in the photic zone of oceans (Noji 1991, Noji *et al.* 1991,

Poulsen and Kjørboe 2005). Physical disruption also increases the surface area for microbial colonisation, arguably a more important process as it leads to mineralization of the faecal material (Povero *et al.* 2003). Micro-organisms involved in degradation of marine copepod faecal pellets are internal to the pellet matrix and survive passage through the invertebrate gut (Turner 2002).

The microbial degradation of faecal pellets is important in oceans and the organic matter from pellets is a significant resource for the microbial loop (Jumars *et al.* 1989, Lampitt *et al.* 1990). Faecal pellet organic matter is mainly mineralized by micro-organisms in the upper parts of the water column, and this reduces the flux of material to the ocean floor (Turner 2002). The microbial loop is also important in streams and rivers where micro-organisms (such as bacteria) recycle particulate and dissolved organic matter (Meyer 1994), and are thought to be a major source of nutrition for detritivores (Cummins and Klug 1979).

Faecal pellets are a component of organic aggregates such as marine snow, the formation of which creates larger particles than the faecal pellets themselves which sink and are broken down more quickly than their component particles (Alldredge and Silver 1988). Binding materials such as exopolymers (EPS) are central to the formation of such aggregates (Wotton 2005).

In chalk streams, faecal pellets are typically found in organic sediments on, and in, the substratum (Ladle and Griffiths 1980, Wharton *et al.* 2006), and also in suspension (Warren *et al.* 2005). Over time, faecal pellets are broken down releasing the organic matter the pellets contain, and having a significant impact on organic matter flux within the stream. These processes are likely to affect the utilisation of organic matter by both

the macroinvertebrate and microbial communities within the stream. In this chapter the binding and breakdown of *Gammarus pulex* faecal pellets in chalk streams is investigated to understand how these processes influence the flux of organic matter, especially that from allochthonous sources, bound into pellets.

3.2. Binding Materials Within *Gammarus pulex* Faecal Pellets

3.2.1. Introduction

Invertebrate faecal pellets are either bound by a covering of peritrophic membrane, internal EPS, or a combination of the two (Wotton and Malmqvist 2001, Wotton 2005). Peritrophic membranes are found in many groups of arthropods and perform a number of functions including protecting the gut wall and regulating the transport of enzymes (Terra 2001). Peritrophic membranes are constantly being secreted, and are often egested with the faeces, forming a wrapping which binds the contents together (Terra 2001). Inside this wrapping, sticky EPS bind organic matter particles together (as well as any other particles in the faecal pellet such as mineral grains). These EPS are produced both in the invertebrate gut and by a wide variety of micro-organisms (such as algae and bacteria) in aquatic environments, and are often found free in the environment as transparent exopolymer particles (TEP) which are often ingested by aquatic organisms (Wotton 2005).

Without binding of any kind, faecal matter would disperse in the water column, but because of binding materials faecal matter is formed into distinct pellets or strings (depending on the taxon) which are physically stable. Because of this, intact pellets are abundant in many aquatic environments including the open ocean (Wassmann *et al.* 1999), benthic marine sediments (Giles and Pilditch 2004) and chalk stream sediments (Ladle and Griffiths 1980). The binding of organic matter into faecal pellets changes their fate in the environment. In chalk streams, sedimentation and retention of organic matter is increased by faecal pellet production as pellets are larger and more dense than their

constituent particles, and as such less easily transported (Ladle and Griffiths 1980, Wotton and Malmqvist 2001).

Using microbiological stains I investigated the materials binding *Gammarus pulex* faecal pellets together, hypothesising that pellets would be bound both by internal EPS and an external peritrophic membrane., in order to determine they remain cohesive organic aggregates in chalk stream ecosystems.

3.2.2. Methods

Production of Faecal Pellets in the Laboratory

Gammarus pulex were collected from the field and kept in aerated aquarium tanks 25 cm long x 15 cm wide x 15 cm deep. They were fed on laboratory-conditioned Horse Chestnut leaves, these being more nutritious to shredders such as *G. pulex* as they contain higher amounts of protein from colonising micro-organisms (e.g. bacteria, fungi). *G. pulex* are known to prefer conditioned leaves (Kaushik and Hynes 1971), and Horse Chestnut leaves improve the survival of *G. pulex* in culture over other types of leaf such as Alder (McCahon and Pascoe 1988a, McCahon and Pascoe 1988b). Leaves were conditioned by placing them in a tank of chalk stream water aerated with a pump for several weeks until they had grown microbial biofilms on their surface.

G. pulex were contained within a 1 mm mesh net suspended 2 cm above the bottom of the tank. This mesh prevented animals from coming into contact with the faecal pellets they produced and causing disruption by their movements and through coprophagy. Faecal pellets were collected from the tanks at regular intervals using a disposable pipette.

EPS in the Pellet Matrix

Faecal pellets were stained to examine them for the presence of EPS using Alcian Blue solution. Pellets were immersed in Alcian Blue solution at a concentration of 10 g l^{-1} (3% acetic acid) for 20 minutes to allow the stain to permeate the pellet matrix. Following this, pellets were washed repeatedly in a succession of 2 litre beakers of distilled water to remove the excess stain. Once washed, pellets were squashed on microscope slides and the presence of EPS observed under low power (x10) light microscopy. EPS appeared bright blue due to staining, whereas organic detritus in the pellets was not stained and remained brown.

Peritrophic Membranes Surrounding the Pellet Matrix

Peritrophic membranes surrounding faecal pellets were visualised using the fluorescent stain Calcofluor White which stains chitin within the membranes. Stained peritrophic membranes fluoresce light blue under a UV light source. Fresh pellets were immersed in Calcofluor White solution at a concentration of 1 g l^{-1} (aqueous) for 20 minutes, removed and washed as above for the Alcian Blue treatment. Pellets were placed on microscope slides and viewed under a low power (x10) fluorescence microscope using a short wave band pass filter (340 - 380 nm excitation, 425 nm emission).

Loss of Peritrophic Membranes From Faecal Pellets

Groups of faecal pellets of increasing age were collected to see if the peritrophic membrane of *G. pulex* pellets is lost soon after egestion or if they remain intact for longer periods. Pellets were produced and allowed to age for 2, 3, 4, 6, 16, 24 and 48 hours at

20°C in water collected from a chalk stream. After ageing pellets were stained using Calcofluor as above and observed under a fluorescence microscope to see if the peritrophic membrane was present or absent. A total of 90 faecal pellets were sampled at each time period.

3.2.3. Results

Observations showed that EPS were found extensively throughout the matrix of *Gammarus pulex* faecal pellets, as areas stained by Alcian Blue were observed. Pellets were also wrapped in a peritrophic membrane on egestion, further binding the organic matter within the pellet. Peritrophic membranes degraded with time however (Figure 3.2.1) and were lost within days of egestion. After 2 hours ~50% of pellets had no covering of peritrophic membrane and after 48 hours post egestion ~90% of faecal pellets had lost it (Figure 3.2.2). As *G. pulex* faecal pellets remain intact for long periods (up to 80 days – Section 3.5), and as the peritrophic membrane is lost early in the majority of pellets, EPS must be responsible for binding over long periods (48 hours or more).

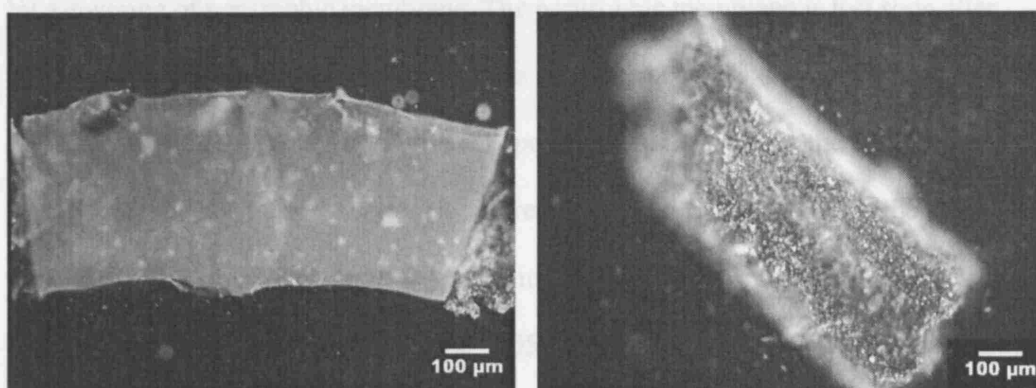


Figure 3.2.1. – Images showing *Gammarus* faecal pellets A) soon after egestion with peritrophic membrane and B) after 48 hours with no peritrophic membrane.

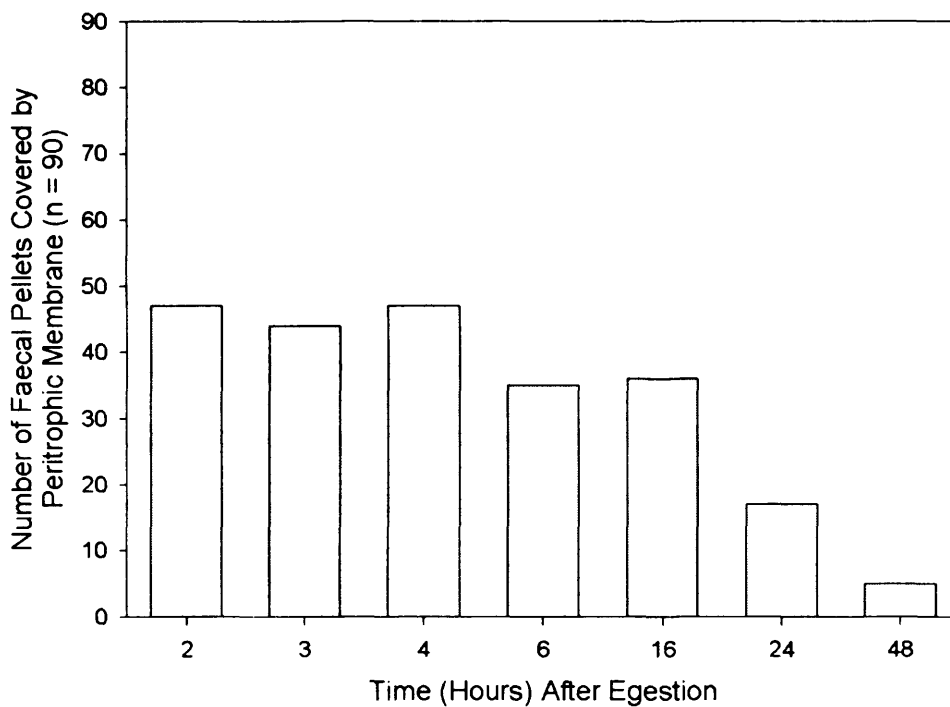


Figure 3.2.2. – Percentage of faecal pellets covered with peritrophic membrane from 2 to 48 hours post-egestion (n = 90).

3.2.4. Discussion

On egestion *Gammarus pulex* faecal pellets are bound internally by EPS and externally by a covering of peritrophic membrane. The peritrophic membrane is lost soon after egestion (1-2 days) and long term binding is facilitated by EPS. *G. pulex* faecal pellets therefore provide a contrast to marine copepod faecal pellets where the peritrophic membrane remains intact for a week or more (Honjo and Roman 1978, Bathelt and Schelske 1983) and where the faecal pellet matrix breaks down rapidly once the membrane is lost (Poulsen and Kiørboe 2005). The peritrophic membrane surrounding copepod faecal pellets is essential for pellet integrity whereas in *G. pulex* faecal pellets it is not.

The internal binding of faecal pellets is very strong and, in laboratory tests, *G. pulex* pellets in water remained intact when exposed to strong hydrodynamic forces produced by magnetic stirrers, bench vortex mixers and even low level sonication. In streams, *Simulium* faecal pellets, that are similarly bound by EPS and possess no peritrophic membrane (Luke Warren, personal communication), traverse torrential rapids without disruption (Malmqvist *et al.* 2001). Although not readily disrupted physically, faecal pellets are changed by micro-organisms such as bacteria and fungi (Bärlocher 1981, Hansen *et al.* 1996, Wotton and Malmqvist 2001) and this is likely to alter the internal binding of the pellet.

3.3. Transformation of Organic Matter Within *Gammarus pulex* Faecal Pellets

3.3.1. Introduction

As time progresses, faecal pellets are broken down by both physical processes (e.g. hydrodynamic forces) and biological processes (e.g. microbial breakdown). Physical breakdown of faecal pellets in the ocean (e.g. by copepod grazing) leads to disintegration of the pellet matrix, releasing the particles they contain and causing an overall shift towards smaller particles in the water column. Particle size can also be increased by breakdown processes, such as when the pellet matrix is only partially disrupted and binding materials are loosened by physical processes (Noji *et al.* 1991), and when micro-organisms break down the pellet matrix from within (Hansen *et al.* 1996).

As previously described in Section 3.2., faecal pellets are primarily bound by EPS. During breakdown of faecal pellets, micro-organisms (such as bacteria) secrete EPS into the pellet matrix and this alters the patterns of binding within the pellet. Over time, the pellet matrix is transformed, and the size-spectrum of particles inside the degraded faecal pellets changes. Microbial breakdown of faecal pellets is therefore an important long-term mechanism for particle size change.

I investigated the effect of microbial breakdown on the size of particles within the *Gammarus pulex*, hypothesising that, over time, microbial processes within the pellet would cause an increase in particle size due to EPS released into the pellet matrix and rebinding organic matter. This experiment aims to show that microbial processes transform the size of organic matter within pellets and that this is likely to affect organic matter transport within chalk streams.

3.3.2. Methods

As faecal pellets degrade and micro-organisms secrete EPS into the pellet matrix, the binding of organic matter there is re-organised. New aggregates are formed, termed here as “Secondary Aggregates” (SecAggs). The bonds within SecAggs are stronger than those between them as within SecAggs binding materials are newer and stronger than in the rest of the pellet matrix. As these new bonds are created and SecAggs form, the mean size of particles within the pellet matrix increases, reflecting the new binding patterns. These particles are then released from the faecal pellet matrix when it breaks down or is disrupted.

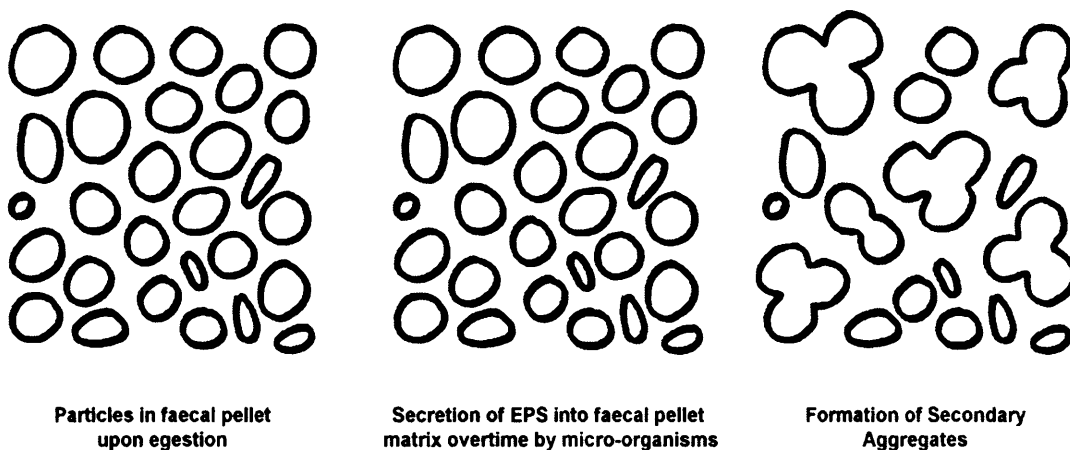


Figure 3.3.1. – A conceptual model of the formation of SecAggs within faecal pellets. As micro-organisms degrade the pellet matrix, EPS is released and this rebinds organic matter particles into larger aggregate particles – SecAggs.

To investigate the formation of SecAggs, individual faecal pellets generated from laboratory *Gammarus pulex* cultures fed on conditioned Horse Chestnut leaves (see page 81) were disrupted in water-filled centrifuge tubes using a bench vortex mixer. Although the mixer provides a strong hydrodynamic force, this was not enough to fully disrupt the

strongly bound faecal pellets, and 0.5 g of glass beads (~0.2mm diameter) were added to impact the pellets during vortexing. This was enough force to disrupt the pellet matrix, but not its smaller components such as SecAggs.

Faecal pellets were spun in the vortex mixer for 1 minute, after which the resulting suspension of particles was filtered through a Whatman 5 µm pore-size polycarbonate membrane filter using a Buchner flask. A glass sinter was used to give even distribution of particles on the membrane. Filters were photographed using a high resolution (8 mega pixel) digital camera and the images analysed using ImageJ (see Appendix B). The average size (as apparent surface area or ASA in mm²) of the SecAggs produced from each pellet was calculated. This procedure was repeated for 24 faecal pellets.

The above treatment was carried out with both recently egested (Fresh) faecal pellets and pellets aged for 30 days at 20°C (Conditioned) to see what effect microbial breakdown has on SecAggs within the pellets and whether, over time, the binding of organic matter is changed within *G. pulex* faecal pellets, as would be expected from the model in Figure 3.3.1. As a control, the above procedure was carried out using iron filings of a similar size to *G. pulex* faecal pellets, and these were not disrupted. The number of observed particles did not change after iron filings were spun in the vortex mixer, demonstrating that all observed particles were released from disrupted faecal pellets and were not added during preparation or by the equipment used.

3.3.3. Results

Conditioned faecal pellets produced significantly larger SecAggs than fresh faecal pellets after disruption ($F_{1,47} = 83.78$ $p < 0.001$ in ANOVA, see Figure 3.3.1.). The size of

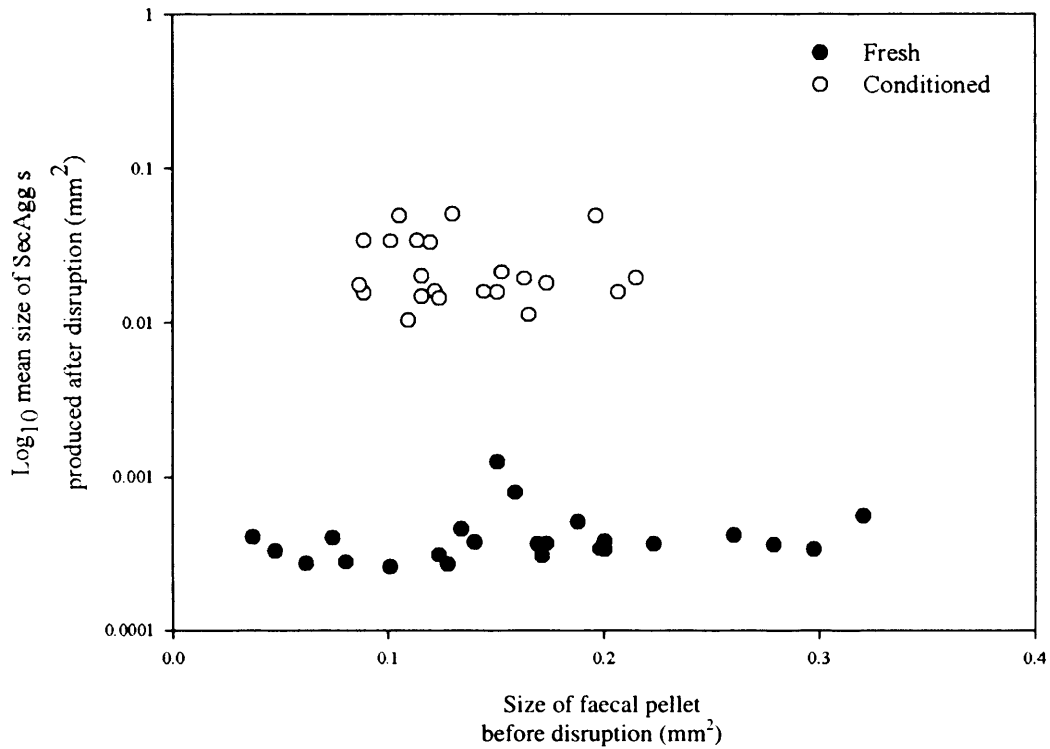


Figure 3.3.2. – The size of SecAggs produced after the disruption of Fresh (closed circles) and Conditioned (open circles) *G. pulex* faecal pellets.

SecAggs produced when freshly-egested pellets were disrupted was $\sim 0.0005 \text{ mm}^2$, but when allowed to condition for 30 days the size of SecAggs produced was two orders of magnitude larger at $\sim 0.05 \text{ mm}^2$. Allowing faecal pellets to condition thus causes a change in the size particles within the faecal pellet matrix, and shows that larger SecAggs of these particles form overtime.

3.3.4. Discussion

Microbial breakdown has a marked effect on the size of components within faecal pellets. Over time, particles within faecal pellets form secondary aggregates, increasing

the average size of particles in the pellet matrix by two orders of magnitude. Micro-organisms such as bacteria secrete EPS and these sticky exudates attach to organic matter particles and bind them together creating SecAggs. The more EPS that is secreted into the faecal pellet matrix, the more binding that can occur and the larger the SecAggs produced.

Gammarus pulex shreds its food into fine particles which are bound by both EPS and a peritrophic membrane. These fine particles are relatively loosely bound when first egested, and it takes a short time for stronger binding to become established by the addition of further EPS to the pellet matrix by micro-organisms. During this early period, the peritrophic membrane provides some integrity to the pellet before the membrane is lost. Binding between particles gets stronger as microbial action proceeds (and the pellet degrades), leading to formation of larger and larger SecAggs.

Increases in particle size such as the formation of SecAggs influences the retention of organic matter in chalk streams by altering particle sinking rate. As such, larger particles are more likely to sediment out rather than be transported downstream, promoting retention of organic matter locally within stream reaches. In oceans, aggregation of particulate organic matter (such as that from the faecal pellets copepods and other pelagic invertebrates) increases the sinking rate of organic matter and therefore rate of loss from the photic zone, removing organic matter from surface waters (Turner 2002).

3.4. The Role of Bacteria in *Gammarus pulex* Faecal Pellet Breakdown

3.4.1. Introduction

Bacteria are the main agents of the breakdown of invertebrate faecal pellets (Hargrave 1976, Hansen *et al.* 1996), but other micro-organisms such as fungi are also involved (Bärlocher 1981). Bacteria are found in large numbers attached to, and contained within faecal pellets in aquatic environments, for example marine copepod faecal pellets have large numbers of associated bacteria, and they often form “lawns” over the surface of the pellet (Turner 2002).

Bacteria are either present within the faecal pellet matrix upon egestion (after surviving gut passage) or colonise from the environment over time (Wotton and Malmqvist 2001). In copepod faecal pellets internal bacteria are the main agent in breakdown of the pellet matrix (Turner 2002), but bacteria in the water column also actively seek out and colonise pellets by following the DOM they release (Hansen and Bech 1996). Faecal pellets are hotspots of microbial respiration in the water column of the ocean (Thor *et al.* 2003), and bacteria within copepod pellets show a rapid increase in metabolism soon after egestion (Olsen *et al.* 2005). This is probably because internal bacteria utilise labile organic materials present in faecal pellets, such as DOM left over from digestion in the invertebrate gut, and are packed into a mass of potential substrates. Colonising bacteria are not able to take advantage of these resources as quickly or as effectively, and as such the importance of internal bacteria increases the recycling of organic matter by the microbial loop. Similar processes may occur in streams and rivers, as these environments also have a well developed microbial loop that quickly utilises available DOM (Meyer

1994) and, as such, bacteria internal to freshwater faecal pellets in streams and rivers may be important components of the microbial loop there. Faecal pellet production by abundant invertebrates (such as *Gammarus pulex*) may therefore stimulate the microbial loop in chalk streams and promote mineralization and recycling of organic matter.

This experiment aims to demonstrate that bacteria play a pivotal role in the degradation of faecal pellets. I hypothesised that, as in marine systems, internal bacteria are responsible for the break down of *G. pulex* faecal pellets. The location and type of micro-organisms breaking down faecal material has consequences for how material is transformed and utilised in the ecosystem and as such determining these factors is important for understanding the processes that recycle faecal pellets in stream ecosystems.

3.4.2. Methods

Measuring the Rate of Faecal Pellet Breakdown

As faecal pellets degrade, their shape changes and this can be used to measure the rate of breakdown that is occurring to the pellet matrix. As pellets increase in age, the binding materials within the pellet matrix are broken down and rearranged, the pellet matrix collapses under gravity and, when viewed from above, faecal pellets appear to increase in surface area. I used this increase in “apparent surface area” (ASA) to track changes within faecal pellets caused by microbial processes. Similar techniques have been used to measure the rate of breakdown of bivalve faeces (Giles and Pilditch 2004).

Faecal Pellet Production and Incubation

Gammarus pulex faecal pellets were produced from laboratory cultures fed conditioned Horse Chestnut leaves. Freshly egested pellets were placed into clear plastic tissue culture trays having 24 x 1.5 cm diameter wells filled with chalk stream water, with 1 pellet per well. The trays were covered with lids and incubated at 20°C.

Experimental Treatments

To ascertain whether bacteria involved in faecal pellet breakdown are internal to the pellet (and thus surviving gut passage) or colonising from the environment, an experiment with five treatments was designed. Five trays were used, each receiving a single treatment :

Treatment 1 - sterile (autoclaved) faecal pellets in sterile (autoclaved) water with antibiotic ($25 \mu\text{g L}^{-1}$ Gentamicin - Wotton *et al.* 1997)

Treatment 2 - untreated (fresh) faecal pellets in untreated (fresh) stream water

Treatment 3 - sterile (autoclaved) faecal pellets in untreated (fresh) stream water

Treatment 4 - untreated (fresh) faecal pellets in sterile (autoclaved) stream water

Treatment 5 - untreated (fresh) pellets in untreated (fresh) stream water with antibiotic ($25 \mu\text{g L}^{-1}$ Gentamicin)

In Treatment 1 there should be no microbial activity, whereas Treatment 2 represents natural conditions with all associated microbial activity. Any breakdown in Treatment 3 results from microbes that are colonizing from the stream water, and in Treatment 4 from

microbial activity within pellets. Treatment 5 represents natural conditions where bacterial activity has been suppressed, and provides a control for Treatment 2 and the untreated components of Treatments 3 and 4. It also shows whether the micro-organisms responsible for breakdown of faecal pellets are primarily bacteria, because Gentamicin does not suppress fungal communities (Taplin 1965, Mukerji and Rao 1968).

The increase in apparent surface area (ASA) of faecal pellets over time was measured by image analysis using ImageJ (see Appendix B). Trays were placed on top of a light box to create maximum contrast between the dark faecal material and the clear background. Images of each faecal pellet were captured using a PC linked CCTV camera every 3 days for 35 days in total.

Data Analysis

To analyse the data collected, the change in surface area at each time point (relative to its size at day 0) for each replicate pellet was calculated. This was done by subtracting the surface area of each pellet at the start of the experiment (day 0) from the surface area of each pellet at each subsequent time interval. Repeated measures ANOVA (and subsequent Tukey-Kramer *post-hoc* test) was then used to test for significant differences between the changes in surface area in each of the five treatments.

3.4.3. Results

Significant differences were found between the five experimental treatments ($F_{4,54} = 88.79$ $p < 0.001$ in repeated measures ANOVA) and *Gammarus pulex* faecal pellets showed two distinct patterns of breakdown during the experiment (Figure 3.4.1.). The

sterilised control (Treatment 1) showed no increase in ASA over time. Fresh pellets in fresh stream water (Treatment 2) showed large increases in ASA over the 35 days and were significantly different from sterilised controls ($p < 0.001$ in Tukey-Kramer *post-hoc* test). This indicates that microbial activity is highly significant in faecal pellet breakdown. The change in ASA of autoclaved faecal pellets in fresh stream water (Treatment 3) was not significantly different to sterilised controls ($p > 0.05$ in Tukey-Kramer *post-hoc* test), and breakdown was therefore halted by inactivation of the microbial community within the pellet. Autoclaving the stream water and leaving the pellets fresh (Treatment 4), failed to halt breakdown and ASA increased over time, being significantly different from sterilised controls ($p < 0.001$ in Tukey-Kramer *post-hoc* test).

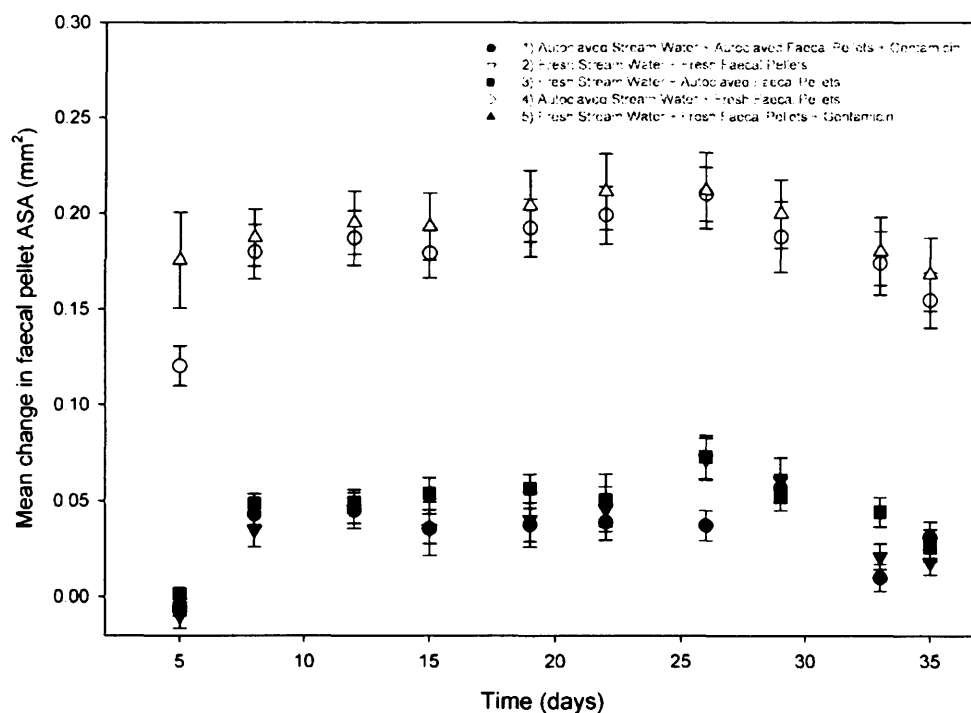


Figure 3.4.1. – Change in apparent surface area with time of *G. pulex* faecal pellets. Each data point represents the mean apparent surface area of all replicates at each time point, and whiskers represent the standard error of the means.

Increases in ASA in this treatment (Treatment 4) were not significantly different from the fresh control (Treatment 2 - $p > 0.05$ in Tukey-Kramer *post-hoc* test).

These results demonstrate that micro-organisms internal to *G. pulex* faecal pellets are responsible for breakdown and not those colonising from the stream water. Addition of gentamicin to fresh pellets and fresh stream water (Treatment 5) halted breakdown and ASA did not increase significantly. ASA was not significantly different to the sterilised controls ($p > 0.05$ in Tukey-Kramer *post-hoc* test). This indicates that the primary agent responsible for breakdown of *G. pulex* faecal pellets are bacteria.

3.4.4. Discussion

The primary agent involved in breakdown of *Gammarus pulex* faecal pellets are internal bacteria that are mixed into the pellet matrix. During gut passage, bacteria are exposed to a variety of hostile conditions, including the presence of digestive enzymes and changes in pH, and this changes the behaviour of bacteria. For example *Legionella* bacteria that survive passage through protistan food vacuoles have increased levels of activity (Atlas 1999). In faecal pellets, bacteria also show increased levels of activity after passage through the gut (Olsen 2005) and this will increase the overall rate of bacterial breakdown of faecal pellets, as breakdown starts as soon as the pellet has been egested. The hostile conditions in the gut stimulate bacteria to produce EPS as a defence, a common response of bacteria to hostile environmental conditions (Hahn *et al.* 2004, Wotton 2005). This explains the abundance of EPS in the pellet matrix.

The internal location of bacteria within faecal pellets is therefore critical to the processes of binding and organic matter transformation I have described. Colonising

bacteria take time to build up numbers and lack the increased activity of bacteria that have passed through the invertebrate gut, therefore they do not play a significant part in faecal pellet breakdown.

Upon egestion, bacteria quickly utilise labile DOM and POM in the faecal pellet matrix and this is shown by high levels of bacterial metabolism and reproduction (Hansen *et al.* 1996, Povero *et al.* 2003, Olsen *et al.* 2005, Frangoulis *et al.* 2005), and this promotes recycling of organic matter from faecal pellets by the microbial loop. Also, bacteria are of high nutritional value as they are high in protein, and as they increase in number in pellets make them richer in protein than other POM sources (Shepard and Minshall 1981). Faecal pellets therefore increase in nutritional value relatively quickly after egestion due to their active internal bacterial community.

3.5. Longevity of *Gammarus pulex* Faecal Pellets and the Influence of Temperature

3.5.1. Introduction

Faecal pellets transport and store significant amounts of organic matter both in the water column and benthic sediments in streams and rivers (Wotton *et al.* 1998, Wotton and Malmqvist 2001, Wotton 2006). The time it takes faecal pellets to break down and release this material is important to the recycling of organic matter within the aquatic environment. If faecal pellets remain intact for long periods (months), organic matter is stored and subjected to microbial transformation such as by the addition of EPS to the pellet matrix (as described in Section 3.3.). If the breakdown of pellets occurs quickly (days to weeks), organic matter is not stored as effectively, and transformation processes will not have time to take effect. Longevity is therefore an important factor when considering the organic matter flux from faecal pellets.

Temperature plays an important role in determining the breakdown rate of faecal pellets in the environment. Although bacteria (the primary agents of faecal pellet breakdown) are optimised for specific temperature ranges, in general increases in environmental temperature increase bacterial growth and activity (Stanier *et al.* 1987). Increases in temperature therefore increase the rate of faecal pellet degradation by increasing bacterial activity within them.

In this experiment, I hypothesised that an increase in temperature would increase the rate of faecal pellet breakdown. I observed *Gammarus pulex* faecal pellets for 80 days at 3 different temperatures to see if temperature was a significant factor influencing the longevity of faecal pellets in chalk streams.

3.5.2. Methods

Faecal pellets were produced from laboratory *Gammarus pulex* fed on conditioned Horse Chestnut leaves. Faecal pellets were collected after 24 hours using a pipette and placed into clear plastic tissue culture trays with 24 x 1.5 cm diameter wells filled with chalk stream water, with 1 pellet per well, giving 24 replicate pellets per tray. To investigate the influence of temperature on faecal pellet breakdown rate, 3 trays stocked as above were placed at different temperatures, one at 4°C, one at 10°C and one at 20°C. Images were taken of each pellet using a CCTV camera linked to a PC every 3-4 days for a total of 80 days and analysed using ImageJ (see Appendix B). This length of time was needed to see if faecal pellets survived for long periods (months) or broke down quickly (days to weeks). The change in ASA of each faecal pellet was measured as described in Section 3.4.2. along with changes in the average number of fragments in the 24 wells. This gave a measure of whether faecal pellets fragmented, and whether their integrity is maintained over long periods. Differences between temperature treatments were analysed using general linear model ANOVA as described in Section 3.4.2.

3.5.3. Results

As temperature increased, the rate of degradation of *Gammarus pulex* faecal pellets increased (Figure 3.5.1.) and there were significant differences between temperature treatments ($F_{2,47} = 50.09$ $p < 0.001$ in repeated measures ANOVA). Pellets incubated at 4°C and 10°C showed little change in ASA and were not significantly different from one another ($p > 0.05$ in Tukey-Kramer *post-hoc* test), but were significantly different from pellets incubated at 20°C (both comparisons $p < 0.001$ in Tukey-Kramer *post-hoc* test).

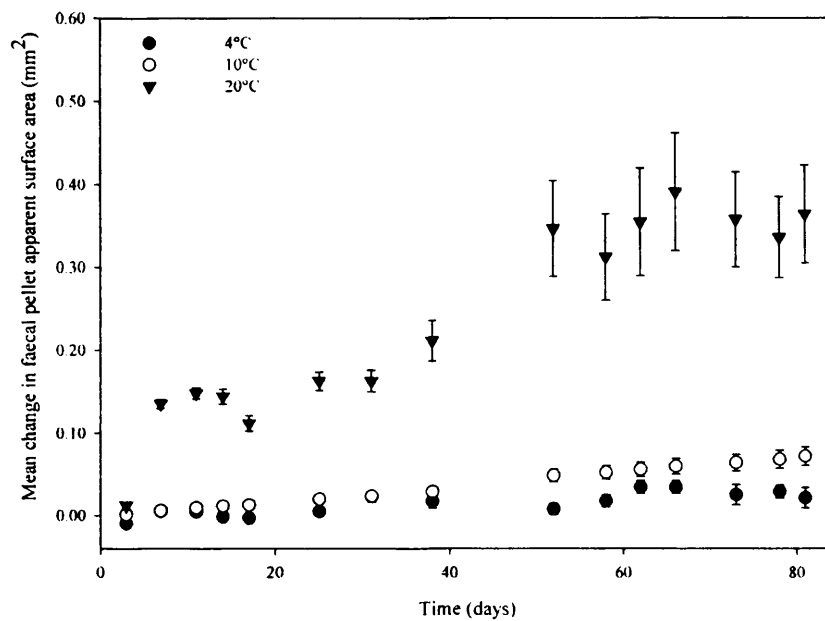


Figure 3.5.1. – Mean change in apparent surface area (ASA) with time for *G. pulex* faecal pellets incubated at 3 different temperatures. Whiskers indicate standard error of the mean.

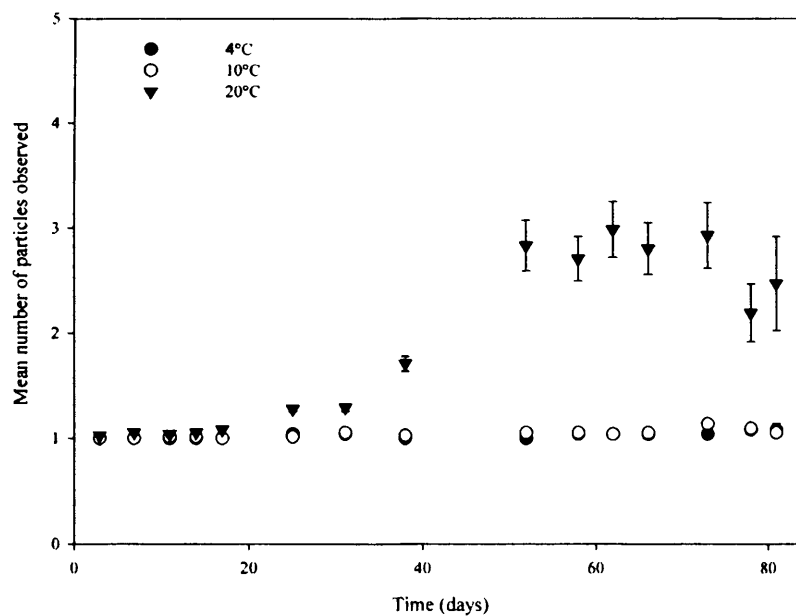


Figure 3.5.2. – Changes in the mean number of particles observed in each well of tissue culture trays containing *G. pulex* faecal pellets at 3 temperatures with time. At 0 days pellets had not fragmented, and as such the number of particles was 1. Whiskers indicate standard error of the mean.

Only *G. pulex* faecal pellets incubated at 20°C showed fragmentation over the 80 days of the experiment (Figure 3.5.2.). Pellets incubated at the lower temperatures did not fragment and remained intact. Overall, there were significant differences in mean number of observed fragments between the three temperature treatments ($F_{2,47} = 17.59$ $p < 0.001$ in repeated measures ANOVA). There were no significant difference in mean number of fragments between 4°C and 10°C ($p > 0.05$ in Tukey-Kramer *post-hoc* test) as the faecal pellets in these treatments did not break up, and the mean number of fragments observed remained at ~1. Both these temperature treatments differed significantly from pellets incubated at 20°C (both $p < 0.001$ in Tukey-Kramer *post-hoc* test) however, and the pellets in this treatment broke up into ~3 fragments after 80 days. Throughout the experiment there was some fluctuation in the mean number of fragments observed in all temperature treatments, and this was due to small fragments rolling underneath the faecal pellet as tissue culture trays were carried around during sampling. Once underneath the pellet these fragments could no longer be observed from above by the camera, and as such the mean number of fragments in the well reduced. If the fragment came into view again, the number increased. Trays were carried carefully to minimise this effect, and the overall trends were easily discernable from this experimental noise.

3.5.4. Discussion

After egestion, *Gammarus pulex* faecal pellets remain cohesive over long periods, retaining the particulate organic matter that is contained in the pellet matrix. Binding of the pellet by EPS is a vital element of this longevity, the materials being held together tightly so that the matrix resists breakdown. *G. pulex* faecal pellets are therefore effective

stores of organic matter, especially in autumn and winter when *G. pulex* feed on leaf fall into the stream (Kaushik and Hynes 1971) and large numbers of faecal pellets are deposited.

However, pellet breakdown is temperature dependant as the rate of degradation increases from approximately zero at below 10°C to pellets showing significant ASA increases and fragmentation at 20°C. The longevity of faecal pellets in the environment therefore depends on temperature. This is a reflection of the role of bacteria within the pellet matrix as at higher temperatures bacteria are able to reproduce faster and their exoenzymes act at a faster rate, increasing the rate of degradation of the pellet matrix and the material it contains. These results indicate that the structure of pellets is stable at lower temperatures but not at higher temperatures. At 10°C and below degradation processes probably don't act at a great enough rate to overcome binding forces within the pellet matrix (both those present from egestion of the pellet and those provided by continuing addition of EPS to the matrix), but at 20°C however degradation processes overcome binding forces and fragmentation occurs.

3.6. The Effect of *Gammarus pulex* Diet on Faecal Pellet Breakdown

3.6.1. Introduction

The food of aquatic invertebrates affects the physical characteristics and composition of their faecal pellets, and this alters how they are transported and broken down in the environment. Changes in diet (both composition and concentration) of marine copepods alters the sinking rate and size of their faecal pellets (Taghon *et al.* 1984, Dagg and Walser 1986, Butler and Dam 1994), and this consequently influences how the organic matter they contain is recycled within the photic zone of oceans. In chalk streams, diet also affects the sinking rate of faecal pellets, as those produced by black flies sink more quickly when mineral grains make up a large proportion of the particles that they filter from the environment, and consequently their pellets are transported shorter distances downstream (Warren 2006).

As a shredder, *Gammarus pulex* uses a number of different foods but prefers those colonised by bacteria and fungi (Kaushik and Hynes 1971, Petersen and Cummins 1974, Golladay *et al.* 1983, Irons *et al.* 1988). As the composition of faecal pellets is determined by the food a *G. pulex* eats, influencing the substrates that are available for the micro-organisms on and within the pellet, the rate of microbial break down of a pellet should also be influenced by diet.

In a similar experiment to that conducted in 3.5., I observed the rate of breakdown of *G. pulex* faecal pellets produced from a high protein or “high quality” diet when compared with those produced from a low protein or “low quality” diet, hypothesising that pellets produced on a high quality diet would contain more labile organic compounds left

undigested by *G. pulex* and therefore would breakdown more quickly due to higher levels of microbial activity than in a low quality diet.

3.6.2. Methods

Two laboratory cultures of *Gammarus pulex* were set up to produce faecal pellets, one was fed a high quality diet of conditioned Horse Chestnut leaves and the other a low quality diet of conditioned Oak leaves as in Section 2.2.2. (also see Appendix A). Faecal pellets were collected after 24 hours using a pipette and placed into clear plastic tissue culture trays with 24 x 1.5 cm diameter wells filled with chalk stream water, with 1 pellet per well, and incubated at 20°C.

For each diet, 3 trays were stocked with pellets (72 pellets in total), and this gave a total of 6 trays and 144 faecal pellets. Images of each pellet were taken every 3-4 days for 35 days in total using a CCTV camera attached to a PC and increases in the ASA of the pellets over time calculated using image analysis to see if there was a difference in the rate of ASA increase between the two diets. Because increases in ASA indicate degradation of the pellet matrix (Section 3.4.), a higher rate of ASA increase indicates a higher rate of break down of the pellet by micro-organisms. Differences between the two diet treatments were analysed using general linear model ANOVA as described in Section 3.4.2.

3.6.3. Results

Faecal pellets produced from animals fed on Horse Chestnut and Oak diets showed similar changes in ASA over time and therefore showed a similar breakdown rate (Figure

3.6.1.). There was no significant difference in breakdown rate between the two diet treatments ($T_{23} = 1.993$ $p = 0.582$ in paired T-Test) over 35 days. The composition of *G. pulex*'s diet does not therefore influence the rate at which their faecal pellets break down.

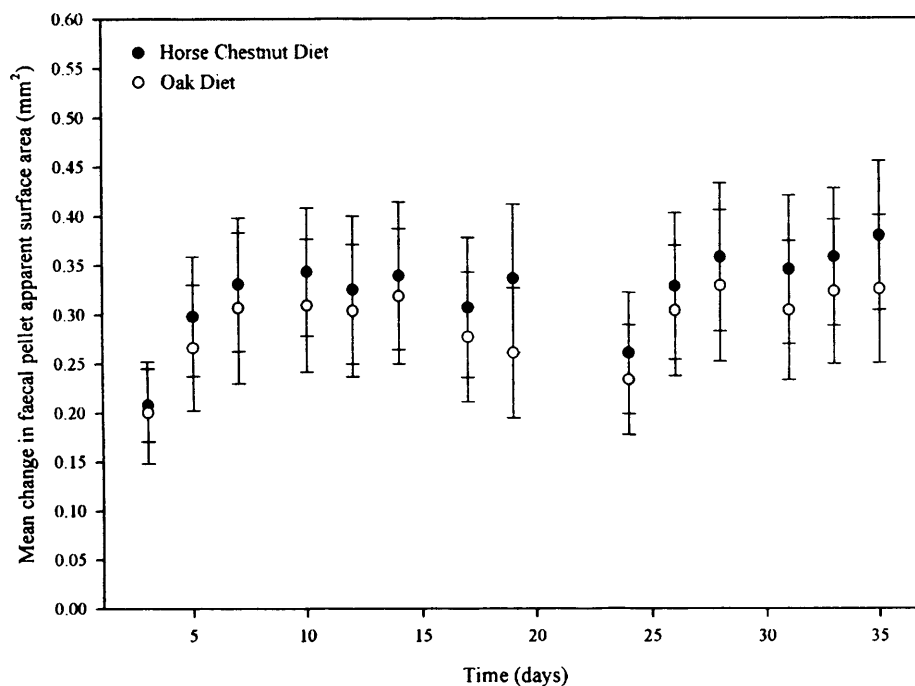


Figure 3.6.1. - Change in apparent surface area (ASA) with time for *Gammarus* faecal pellets produced by *Gammarus* fed Horse Chestnut leaves (closed circles) and Oak leaves (open circles). Whiskers indicate standard error of the mean.

3.6.4. Discussion

Diet is not a significant factor influencing the breakdown rate of *Gammarus pulex* faecal pellets as there was no difference in the rate of ASA increase of faecal pellets fed Horse Chestnut leaves and Oak leaves. Higher quality diets do not cause an increase in breakdown rate. Changes in the diets available in chalk streams to *G. pulex* do not alter the rate at which their faecal pellets break down. This is contrary to evidence from oceans

where the diet of copepods is a determinant of the rate of breakdown of their faecal pellets (Hansen *et al.* 1996).

The assimilation efficiency of *G. pulex* for its food is typically low. Estimates of the assimilation efficiency of *G. pulex* fed conditioned alder leaves made by Nilson (1974) ranged from 30 to 40%, but using the same diet Berrie (1976) reported assimilation efficiency to be lower at between 14 to 26%. Dissolved organic matter leaches from leaves soon after they fall into streams (1-2 days), and micro-organisms will quickly utilise the more labile organic materials in the leaf, leaving only more refractory organic matter such as cellulose and lignin (Kaushik and Hynes 1971, Petersen and Cummins 1974). During conditioning, bacteria and fungi colonise leaves and increase their nutritional value by increasing the amount of protein available to consumers which are thought to gain nutrition primarily from the colonising micro-organisms (Cummins 1974, Cummins and Klug 1979).

When *G. pulex* consumes leaf material, it assimilates the more labile carbohydrates and proteins leaving the refractory cellulose and lignin behind as *G. pulex* cannot digest these compounds (Monk 1977, McGrath and Matthews 2000). As such, faecal pellets from different leaf diets are composed mainly of refractory cellulose and lignin, as other components of the leaf will have been assimilated. In the marine environment the materials that form the matrix of copepod faecal pellets are more diverse and therefore have a greater influence on the rate of break down. In their study of copepod faecal pellet degradation, Hansen *et al.* (1996) attributed the differences in pellet break down rate to physiology of the phytoplankton the copepods fed on. When fed diatoms, copepods produced pellets that degraded more slowly due to the presence of silica frustules from

the diatoms' shell, and these were not broken down by bacteria. Dinoflagellate diets produced pellets that broke down more quickly as bacteria could utilise all of the substrates available in the faecal material.

When feeding on dead leaves in fresh waters (such as chalk streams), *G. pulex* egests faecal pellets mainly composed of undigested cellulose, lignin and other refractory organic materials, no matter which leaf species they are sourced from other more labile organic materials are digested and absorbed. This explains the lack of difference between the two diet treatments, as it will take a similar amount of time for microbial communities in pellets produced from different diets to break down the material in the pellet, as the substrates available will be similar. Over time however, as occurs with leaves, pellets become conditioned by growth of micro-organisms and these add protein to the pellet matrix that increases their nutritional value (Shepard and Minshall 1981). This may influence the utilisation of pellets by promoting a succession of microbial communities within the pellet (Hansen and Bech 1996) or making them a more attractive food item for coprophagous invertebrates.

3.7. Aggregation of *Gammarus* Faecal Pellets and the Influence of Temperature

3.7.1. Introduction

POM in the water column of the oceans, lakes, rivers and streams is known to form aggregates, and binding materials such as EPS facilitate the process of aggregation by sticking the individual particles together. Organic aggregates are composed of a variety of materials including the bodies of planktonic organisms (such as dinoflagellates and diatoms), particulate organic matter and invertebrate faecal pellets (Alldredge and Gotschalk 1990, Turner 2002). Organic aggregates generally form when suspended particles collide and stick together, but are also produced by the feeding actions of planktonic organisms such as Larvaceans (Alldredge and Gotschalk 1988, Wotton 1994, Simon *et al.* 2002). Faecal pellets are another form of organic aggregate but clearly formed by different processes in the invertebrate gut as a result of invertebrate feeding, rather than in the water column by physical processes.

Significant attention has been paid to organic aggregates in marine environments. These aggregates, termed “marine snow” play a key role in nutrient cycling and are centres of microbial activity (Simon *et al.* 2002). Marine snows are hotspots of respiration in the water column (Ploug *et al.* 1999) and because of this the internal environment of the aggregate differs from the water column, having a lower pH and oxygen concentration (Alldredge and Cohen 1987). Organic aggregates also occur in lakes where they are termed “lake snow” (Grossart and Simon 1993) and more recently in rivers where they are termed “river snow” (Bockelmann *et al.* 2000, Neu 2000).

Organic aggregates influence organic matter flux by scavenging smaller particles as they sink or are transported in currents, creating larger aggregates and the transport and storage of the material is altered (Hill *et al.* 1990, Simon *et al.* 2002). For example, phytoplankton cells form aggregates in the later stages of blooms or during the severe mixing created by storms and these aggregates then sink, transporting significant amounts of organic matter from the photic zone to the sea bed (Smetacek 1985).

Bacteria and other micro-organisms are significant producers of EPS which are essential to the aggregation of particles into marine snow, lake snow and river snow (Simon 2002, Wotton 2005). As the metabolism of micro-organisms is dependent on environmental temperature, with increases in temperature increasing metabolism, an increase in temperature may lead to the release of more EPS and promote aggregation.

Here I investigate whether the faecal pellets of *Gammarus pulex* form aggregates when incubated together for long periods of time by hypothesising that, over time, bond between pellets form and as such they become joined into larger aggregates. This information has further implications for how organic matter is transported and stored in chalk streams as faecal pellets because aggregates have altered physical and microbial properties.

3.7.2. Methods

Overview

During aggregation of organic matter, bonds form between organic matter particles and they resist separation by physical forces (such as the hydrodynamic forces in a river). These bonds are formed by complex chemical and physical interactions between the

individual particles (Johnson *et al.* 1994). In *Gammarus* faecal pellets, sticky EPS on the surface of the pellets that may bind other pellets or organic materials, and the growth of fungal hyphae from one pellet to another are possible mechanisms producing aggregates (when *Gammarus pulex* faecal pellets >1 day old are stained with Calcofluor White, fungal hyphae can be observed and where faecal pellets have been in close proximity hyphae emerging from one pellet often penetrate others possibly forming bonds between pellets).

To study whether *G. pulex* faecal pellets form aggregates, the *K*-nearest neighbour algorithm was used. This is a form of pattern recognition often used in geographical research where points in space are classified by their distance from one another. For example, on a map of towns in the UK, the algorithm will calculate the “nearest neighbour distance”, the distance from each town to its closest neighbour. They can then be sorted based on this distance. Here, instead of a map, 2-dimensional images of the distribution of faecal pellets over a known area (the base of a white enamel tray) were used. Nearest neighbour distance was calculated for each faecal pellet (the distance from each pellet to the pellet closest to it). If aggregation of faecal pellets occurred, nearest neighbour distance increased as there are fewer particles spread over the same area. Nearest neighbour distance could also decrease, indicating that pellets have broken apart and resulting in fragmentation.

Faecal Pellet Production and Experimental Treatments

Faecal pellets were produced using laboratory cultures of *G. pulex* fed conditioned Horse Chestnut leaves. Faecal pellets were collected from the tanks after 48 hours of

production so that a large number were available for the experiment. These pellets were suspended in chalk stream water and the volume adjusted so that there was ~50 faecal pellets per ml, this suspension served as a stock for the experiment.

Centrifuge tubes with a volume of 15 ml were filled with 6 ml of this stock (~300 faecal pellets), and the total volume made up to 15 ml with chalk stream water. These were termed “FP Tubes”. 3 replicate groups of 9 FP tubes were prepared (giving 27 in total) and placed on a plankton wheel (a mixer that rotates with a “Ferris Wheel” like action to promote admixture and collision of particles) rotating at 30 rpm. 3 tubes, one from each replicate, were removed from the plankton wheel every 3-4 days and sampled for the nearest neighbour distance between particles. Sampling occurred 0, 1, 7, 10, 14, 17, 21, 24, and 30 days after the start of the experiment.

To control the experiment, a suspension of sand grains at ~50 grains per ml was produced and a complementary set of tubes to those containing faecal pellets prepared. These were termed “Control Tubes”. Sand grains contain no biological component and are inert chemically, thus do not form aggregates under laboratory conditions. Coarse (~1 mm diameter) building sand which was free of biofilms or adsorbed EPS which may have caused aggregation was used. Control tubes were stocked with 6 ml of sand grain suspension (~300 sand grains per tube) and chalk stream water added so the total volume of the tubes was 15 ml. A total of 27 Control Tubes were prepared and for every FP Tube that was sampled, a control tube was sampled as well.

To see if temperature affected the rate of aggregation, two plankton wheels were prepared as above, one was placed at 10°C and one at 20°C. These were started at identical times and tubes sampled on identical days after the start of the experiment.

Sampling for Aggregation Using Image Analysis and Nearest Neighbour Algorithm

When the contents of tubes were analysed for aggregation each tube was sampled individually (the contents of tubes were never mixed). The contents of the tubes were poured into a white enamel tray (10 cm long x 5 cm wide) and gently swirled twice to distribute the particles evenly. A digital image of the distribution of particles in the tray was taken using a CCTV camera linked to a PC and stored for analysis.

Images were analysed using a combination of ImageJ (see Appendix B) and the freeware package Spatial Analysis Utilities (<http://www.archeogr.unisi.it/infapp/sau/>). These computer packages analysed the distribution of faecal pellets in the images and returned the mean nearest neighbour distance of the sample. General linear model ANOVA was used to compare replicates and controls to see if aggregation occurred.

3.7.3. Results

Aggregation at 10°C

There was no significant difference in mean nearest neighbour distance between any of the three replicates and their controls and no overall increase or decrease in nearest neighbour distance was observed (Figure 3.7.1.). There was no significant difference between replicate 1 and control 1 ($F_{1,17} = 3.99$ $p = 0.063$ in ANOVA), replicate 2 and control 2 ($F_{1,17} = 0.33$ $p = 0.574$ in ANOVA) and replicate 3 and control 3 ($F_{1,17} = 0.73$ $p = 0.404$). Therefore, there was no aggregation of *Gammarus pulex* faecal pellets at 10°C.

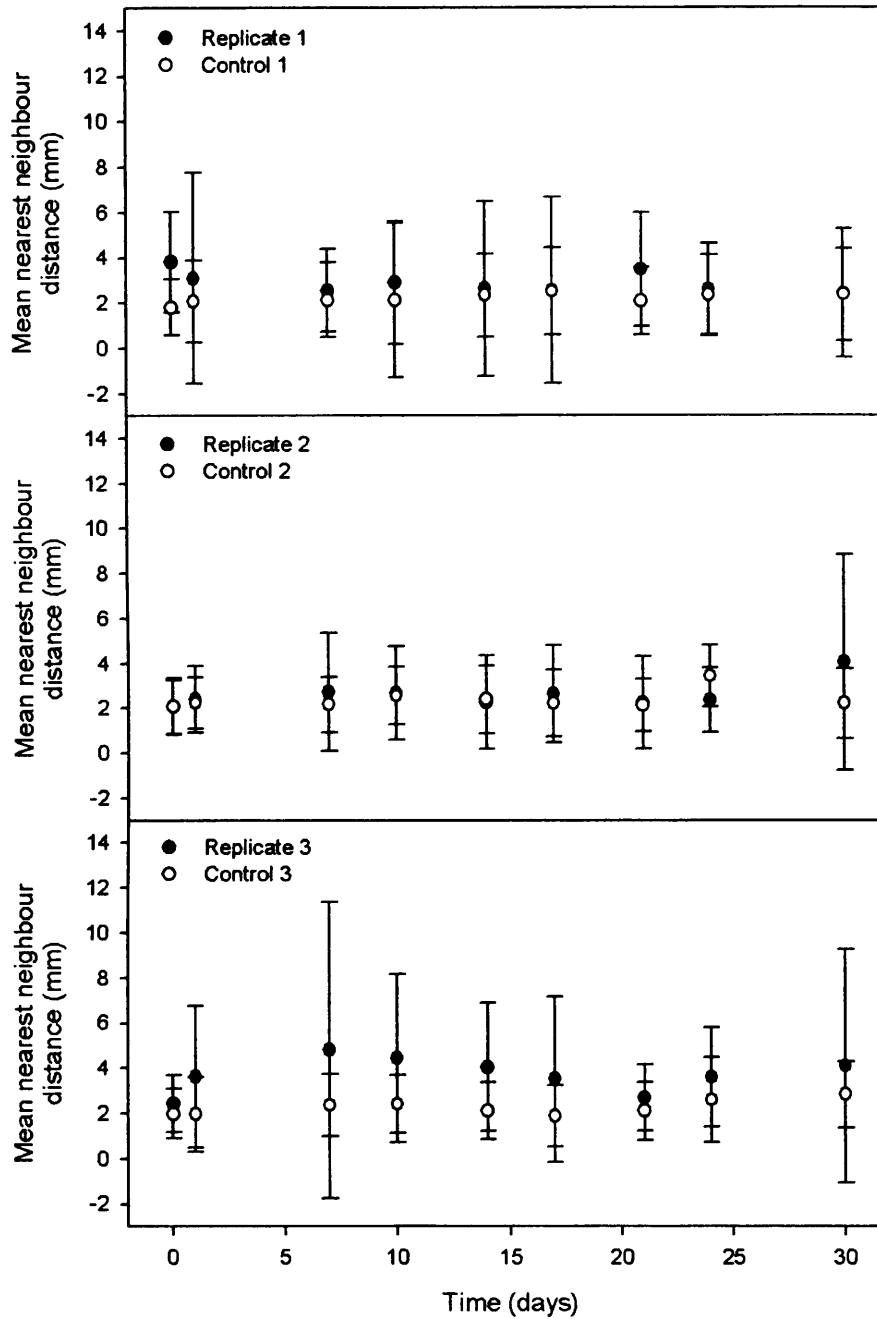


Figure 3.7.1. – Mean nearest neighbour distance of *G. pulex* faecal pellets (closed circles) and sand grain controls (open circles) over time when incubated at 10°C. Whiskers indicate standard error of the mean.

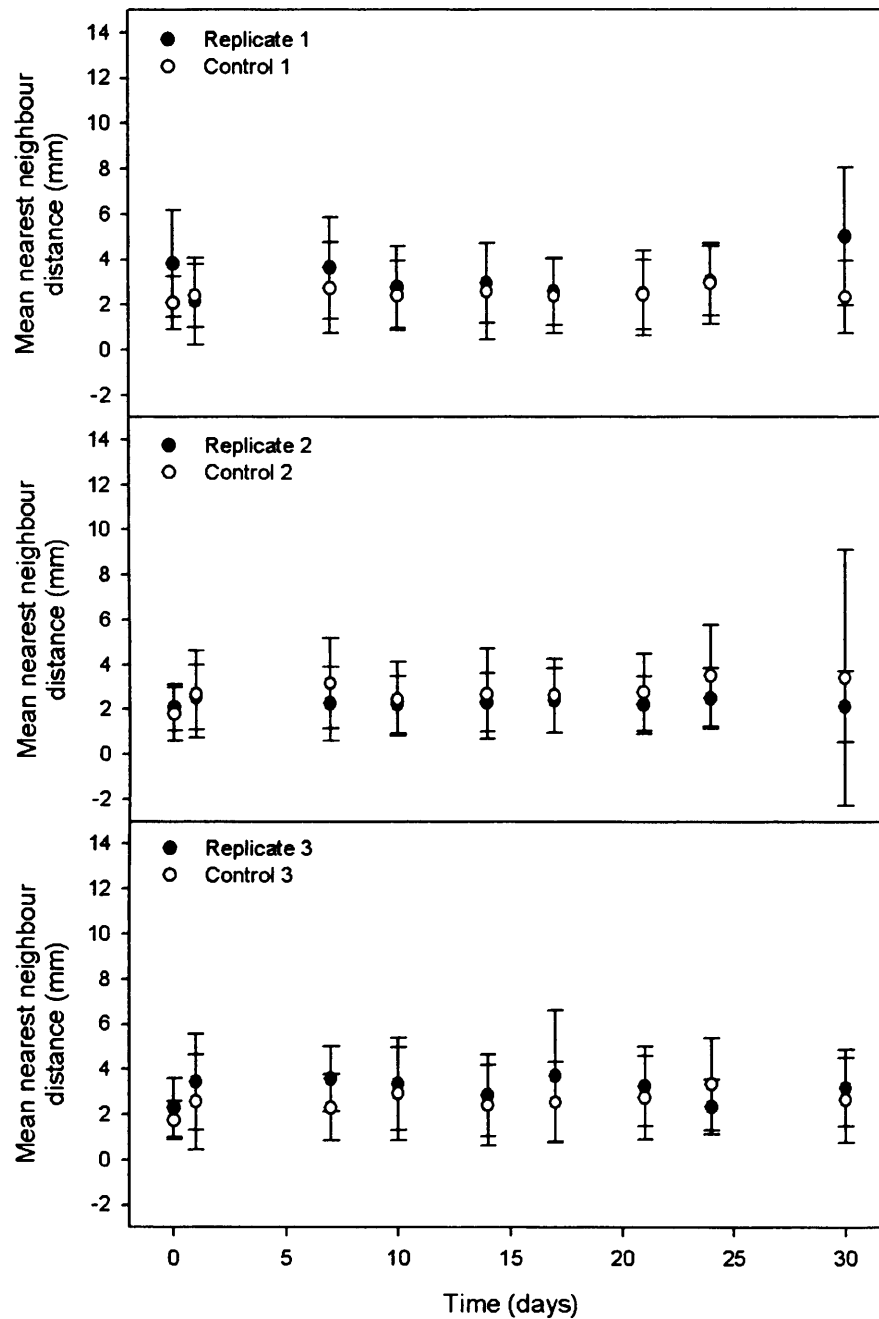


Figure 3.7.2. – Mean nearest neighbour distance of *G. pulex* faecal pellets (closed circles) and sand grain controls (open circles) over time when incubated at 20°C. Whiskers indicate standard error of the mean.

Aggregation at 20°C

Significant differences in mean nearest neighbour difference were found between 2 of the 3 replicates and their controls at the higher temperature, although there was no overall increase or decrease in nearest neighbour distance (Figure 3.7.2.). Both replicate 1 and replicate 3 differed significantly from their controls ($F_{1,17} = 20.77$ $p < 0.001$ and $F_{1,17} = 9.29$ $p < 0.001$ respectively in ANOVA). Replicate 2 did not differ significantly from its control ($F_{1,17} = 1.30$ $p = 0.270$ in ANOVA).

Mean nearest neighbour distance varied widely at this temperature. For example, in replicate 1 (where the replicate and controls were found to be significantly different) mean nearest neighbour distance appears to increase and decrease several times. In this replicate, nearest neighbour distance was lower after 30 days than after 1 day, indicating that no aggregates had formed. A distinct increase in mean nearest neighbour distance is only shown in replicate 3 where values are consistently higher than the controls. However nearest neighbour distance decreased from 7 to 21 days before increasing again.

The significant differences observed in replicates 1 and 3 from their controls represent experimental variation and not aggregation of faecal pellets. As such there is no strong evidence for faecal pellet aggregation over time at 20°C.

3.7.4. Discussion

There was little evidence that *Gammarus pulex* faecal pellets form larger aggregates over time at either 10°C or 20°C as no overall increase in mean nearest neighbour distance occurred over the 30 days of the experiment. During sampling both increases and decreases in mean nearest neighbour distance occurred rather than an overall increase,

and this variation probably explains the significant differences seen between replicates 1 & 3 at 20°C and their respective controls, rather than indicating aggregation had occurred.

It is unlikely that the method used to analyse the samples disrupted any aggregates as the bonds between particles are strong, and resist disaggregation (Alldredge and Gotschalk 1990). Pellets were treated very carefully during preparation and sampling and it is unlikely that aggregates could have been disrupted before they were photographed and analysed. If aggregates did form but were weak enough to be disrupted by the method used here, it is unlikely they would survive in the stream environment as hydrodynamic forces would break the aggregates of faecal pellets apart. Any possible binding mechanisms between pellets, such as by fungi or EPS, are therefore not strong enough to form stable aggregates in the environment.

Organic aggregates such as marine, lake and river “snows” often contain large amounts of faecal material, and this affects their fate in the ecosystem. As aggregates form and increase in size, the sinking rate of the aggregate is altered and this changes how the aggregate (and its constituent organic matter) is transported. The sinking rate of marine snow decreases as its size increases, and aggregation is therefore thought to enhance the residence time of organic matter within the photic zone of oceans (Asper 1987, Alldredge *et al.* 1987, Alldredge and Gotschalk 1988). Lake snow behaves in a similar way (Grossart and Simon 1993). Similarly, as aggregates in streams and rivers increase in size their sinking rate decreases (as with aggregates in the oceans and in lakes) because their porosity increases and density decreases (Droppo *et al.* 1997). In streams therefore, as

particles aggregate, their potential for transport in the current will increase leading to a greater amount of organic matter in transport.

Because *G. pulex* faecal pellets do not form aggregates, organic matter is more likely to remain *in situ* in chalk stream sediments until either the pellet matrix is disrupted by macro-invertebrates (such as deposit feeders) or is degraded by microbial action. These two processes (physical disruption and microbial degradation) release particles smaller than the parent faecal pellets, and therefore any material released into transport will be in this form and not as large aggregates.

3.8. General Discussion

3.8.1. Faecal Pellets as Products of Ecosystem Engineering

The production of faecal pellets can be viewed as a process of “ecosystem engineering” (Wotton and Malmqvist 2001) where organic matter is converted from one form to another by aquatic organisms, and consequently its properties are altered. Although *Gammarus* are classified as shredders under the functional feeding group concept, and dead leaves are quoted as their major food source, dead leaves are a seasonal resource in chalk streams and other temperate streams and rivers. They are only present in large quantities in autumn and early winter and are broken down quickly by a combination of microbial and macroinvertebrate influences (Petersen and Cummins 1974), as well as being easily washed out of the stream by floods. *Gammarus pulex* faecal pellets (which contain large amounts of allochthonous organic matter during autumn and early winter) are found in chalk streams all year round, both within sediments and in the substratum. Because *G. pulex* faecal pellets are long-lived (retaining and storing organic matter), and are not easily transported because they are deposited in the substratum and have a high sinking rate, they increase the residence time of allochthonous organic matter within the stream by preventing its downstream loss.

POM in invertebrate faecal pellets has passed through the gut where digestive enzymes partially break it down, and it is egested mixed with active bacteria and other micro-organisms that have survived gut passage. Binding materials and DOM (Jumars *et al.* 1989) are also present. This makes faecal pellets more than simple collections of

refractory organic matter, but complex micro-habitats with the ability to transform organic matter and alter its transport and storage in the environment.

3.8.2. Longevity and Stability of *Gammarus pulex* Faecal Pellets

Where there is no physical disruption, *Gammarus pulex* faecal pellets are surprisingly stable, remaining intact for 80 days in my experiments, and pellets may remain intact for even longer periods than this. This stability is produced by the abundant EPS found throughout the faecal pellet matrix which tightly binds the organic matter particles together. *G. pulex* is a shredder and chews its food into fine pieces, creating a large surface area for attachment of EPS and leading to the strong bonding that was observed. Other chalk stream invertebrates that feed on dead leaves have less cohesive faecal pellets, for example pellets of the mayfly *Ephemera* sp. which are friable and non-cohesive (Ladle and Griffiths 1980), demonstrating that strong bonding is not a ubiquitous feature of chalk stream invertebrates, and that intact pellets of some organisms will be more dominant in sediments than others. The differences between binding in pellets are likely caused by differences in gut physiology as this determines the shape of the pellets, and the rate at which material passes through the gut (Ladle and Griffiths 1980). For example, the tightly bound pellets of *Simulium* spp. larvae are a product of their compressive hindgut (Chapman 1998).

Microbial action over time reinforces bonding of the pellet matrix. As time progresses, organic matter particles within *G. pulex* faecal pellets are rebound into larger secondary aggregates demonstrating that the bonding of organic matter in pellets is a dynamic process – bonds are constantly being formed and broken down. Bonding by EPS on

egestion is strengthened over time as bacteria start to breakdown the organic matter in the pellet matrix and release EPS into the pellet matrix. The peritrophic membrane provides an initial protection against the disruption of the pellet matrix and allows this to occur.

When temperatures increase to ~20°C pellets begin to fragment due to microbial action, but only after a period of around 30 days which appears to be the time needed for micro-organisms to have a significant effect on the pellet. After this time the stability of the pellet matrix starts to fail. Microbial degradation processes such as the release of exoenzymes by bacteria and fungi probably occur at a greater rate as increases in temperature promote their growth in faecal pellets, especially that of bacteria (Amon and Herndl 1991). This causes break down processes to overcome bonding processes and leads to degradation of the pellet matrix. High temperatures may also change the microbial community of the pellet, as has been shown to occur in soil bacterial communities (Zogg *et al.* 1997), and this new community may either contribute fewer EPS to the matrix or may be able to degrade the pellet more effectively.

Generally, temperatures in chalk streams are relatively low, slowly fluctuating around 10°C, and are mediated by groundwater which typically enters the stream at 10°C - 11°C (Mackey and Berrie 1991, Berrie 1992). A typical annual temperature range for a chalk stream is 5°C - 17°C (Mackey and Berrie 1991). Only in the warmest part of summer, where water temperature can approach 20°C for more than 30 days will there be significant fragmentation of faecal pellets due to microbial processes. In autumn and winter when temperatures are low (5°C - 10°C) faecal pellets will remain intact, and organic matter (especially allochthonous materials) within them will be stored. As

fragmentation occurs only after 30 days, organic matter will have been re-bound by microbial processes into larger secondary aggregates (see Section 3.3).

3.8.3. Transformation of Organic Matter in Faecal Pellets

Organic matter is continuously changed by the process of pelletisation, from egestion of the faecal pellet to the time when it is broken down. Micro-organisms are the mediators of these changes, causing rearrangement of bonding patterns within the pellet matrix as they metabolise the organic matter it contains, and this leads to a transformation in the size of POM within the pellet matrix, causing it to increase over time.

These transformations in particle size and production of Secondary Aggregates within the pellet matrix have important consequences for chalk streams. The larger secondary aggregates observed within 30 day old *Gammarus pulex* faecal pellets are probably less easily transported in the water column than smaller particles released from freshly egested pellets. Formation of secondary aggregates therefore leads to retention of organic matter and the build up of organic sediments even when the pellet matrix has broken down. Transformation processes such as those observed inside *G. pulex* faecal pellets can have significant effects on organic matter in sediments. Drake *et al.* (2002) showed that conversion of fine organic sediments to much larger faecal pellets by Polychaete worms caused an increase in sinking rate of 3 orders of magnitude, increasing sedimentation of organic matter and reducing the potential for transported and re-suspension considerably. An increase in particle size not only increases retention of material locally within stream sediments, but prevents filter feeders (such as blackfly larvae) from accessing the

material as is less likely to be re-suspended and encountered by filter feeders, or the particles may be too large to be utilised if encountered.

Transformation of *G. pulex* faecal pellets is not only physical but chemical. Shepard and Minshall (1981) showed that faecal pellets from invertebrates in a Rocky Mountain stream were enriched with protein compared to other forms of organic detritus. This effect probably increases with time as bacteria reproduce and metabolise the organic matter within the pellet. Protein enrichment increases the nutritional value of faecal pellets to macroinvertebrate detritivores, likely making them a more preferable food resource.

Transformation is an internal process in *G. pulex* faecal pellets however, as larger scale changes involving groups of faecal pellets do not occur. There is no evidence that *G. pulex* faecal pellets form aggregates when they contact one another if in transport in the water column or in sediments, and they probably do not form a large part of river snows which will contain smaller, more easily transported pellets such as those of larval blackflies. Aggregation of *G. pulex* faecal pellets may not occur simply because they are too massive and binding too weak to support them, or it may be that as most bacterial activity occurs within the pellet localising any binding materials there and away from the surface where they could potentially form bonds with other particles.

3.8.4. Faecal Pellet Degradation and the Microbial Loop in Aquatic Environments

Since the description of the microbial loop in marine systems (Pomeroy 1974, Azam *et al.* 1983) the principles underlying the concept have been applied to all types of freshwater ecosystems including streams and rivers (Meyer 1994). Faecal pellets have

become implicated in the microbial loop (Jumars *et al.* 1989, Lampitt *et al.* 1990), being a readily available source of DOM and POM. Faecal pellets are hotspots of microbial respiration in the water column (Ploug *et al.* 1999), and bacteria internal to the pellet matrix are most important in their break down (Turner 2002).

Gammarus pulex faecal pellets are also broken down mainly by internal bacteria, as shown in Section 3.4. It has been noted that the microbial loop may be a more important trophic link in streams and rivers than in marine systems (Meyer 1994), and that carbon from microbial degradation of detritus forms a significant portion of the carbon ingested by invertebrates at many trophic levels (Hall and Meyer 1998). As faecal pellets are so prevalent in the chalk stream environment, especially those of *Simulium* (Warren *et al.* 2005, Wharton *et al.* 2006), and micro-organisms (particularly bacteria) are so heavily involved in their breakdown, faecal pellets are an important source of energy of micro-organisms in chalk streams. They also affect the rate at which organic matter is recycled. Faecal pellets consist mainly of refractory organic matter and are present for long periods, thus they act as an energy bottleneck and regulate the supply of carbon to successive trophic levels (Hargrave 1976).

Chalk streams generally have a stable hydrology, buffered temperature fluctuations and predictable seasonal growth patterns of macrophytes (Berrie 1992). They show high invertebrate biomass for the area they cover, although their overall biodiversity is not exceptional (Wright *et al.* 1984, Berrie 1992). Detritivores dominate the fauna however, suggesting that readily available organic matter, a large portion of which is pelletised, is able to supply energy to a large number of different organisms with different feeding strategies. Of 69 taxa found in chalk streams, 54 can be classified as detritivores, being

either collector-gatherers, collector-filterers or shredders (78%) and probably gain a significant portion of their nutrition from faecal pellets (Pardo and Armitage 1997). Faecal pellets and their associated microbial community represent a stable resource that may be instrumental in supporting the high diversity and biomass of detritivore taxa that is found in chalk streams, and therefore successive trophic levels as well.

3.9. References

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4. Utilisation of *Gammarus Pulex* Faecal Pellets in Chalk

Streams

4.1. General Introduction.

Aquatic micro-organisms and invertebrates use faecal pellets as a food resource (Wotton and Malmqvist 2001), such as in oceans where faecal pellets are a significant substrate for the microbial loop and food for zooplankton such as pelagic copepods (Lampitt *et al.* 1990). Utilisation of faecal pellets by these organisms causes physical alterations to the pellets, significantly reducing their sinking rate and enhancing residence time of organic matter in the photic zone, therefore maintaining its availability to other pelagic organisms (Noji 1991).

As unutilised faecal material sinks to the ocean floor, it collides with marine snow and organic aggregates (such as other faecal pellets) , becoming incorporated into their matrix and forming larger aggregates (Wotton 2005). These sink to the sea bed and become part of the accumulated sediment there, and are fed upon by detritus feeders living in the sediment which constitute the majority of deep sea organisms (Etter and Grassle 1992). Marine faecal pellets are therefore an important source of organic matter not only for pelagic communities in the photic zone, but for sea bed communities as well (Tyler 1988, Turner 2002).

Faecal pellets also provide an organic matter resource to organisms in streams and rivers (Hargrave 1976, Wotton *et al.* 1998). *Simulium* larvae capture the faecal pellets released by individuals upstream and utilise this material as food, assimilating both the undigested organic matter in the pellet matrix (Wotton 1980b) and micro-organisms that colonise the pellets (Fredeen 1964, Wotton 1980a). Invertebrate faecal material is readily utilised by micro-organisms (Bärlocher 1981, Delille and Razouls 1994, Hansen *et al.* 1996), and is a major source of organic matter for organisms of the microbial loop in streams and rivers, providing a significant link to higher trophic levels (Meyer and Ohop 1983, Meyer 1994). In chalk streams large numbers of faecal pellets are stored in sediments and are available all year round (Ladle and Griffiths 1980, Warren *et al.* 2005). As such pellets act to buffer seasonal changes in the availability of other detrital food resources (Hargrave 1976).

The faecal pellets of *Gammarus pulex* are likely to be a resource both for micro-organisms (and therefore the microbial loop) and detritivorous invertebrates, shunting organic matter from allochthonous sources (dead leaves) to other communities in the ecosystem. In this chapter utilisation of *G. pulex* faecal pellets by micro-organisms and chalk stream invertebrates is investigated in order to ascertain whether they are a resource for chalk stream organisms.

4.2. Growth of Bacteria Associated with *Gammarus pulex* Faecal Pellets Under Laboratory and Field Conditions

4.2.1. Introduction

Bacteria are the main agent responsible for the decomposition of *Gammarus pulex* faecal pellets (see Section 3.4.) and their associated organic matter, and are present in the pellet matrix on egestion. Bacteria break down and utilise particulate organic matter in the pellet for growth, decomposing refractory organic matter by exoenzymes and absorbing the resulting organic compounds for metabolism (Chróst 1991, Vrba *et al.* 2004).

Numbers of bacteria in marine copepod faecal pellets increase quickly soon after egestion, and during this time much of the labile organic matter in the pellet matrix is utilised to support growing bacterial numbers (Hansen *et al.* 1996, Olsen *et al.* 2005). Microbial respiration within copepod faecal pellets typically reaches a peak within hours of egestion, after which it decreases sharply and thereafter remains constant (Olsen *et al.* 2005). This indicates that only a small amount of labile material is available and any remaining material is refractory (Hargrave 1976, Olsen *et al.* 2005). Bacterial growth appears to be enhanced on and in copepod faecal pellets, and numbers of bacteria within the matrix of faecal pellets are up to 5 times higher than free-living bacteria in a corresponding volume of sea water (Jacobsen and Azam 1984, Delille and Razouls 1994, Turner 2002, Thor *et al.* 2003).

The growth of bacteria on *G. pulex* faecal pellets is important to understanding how organic matter is transferred from the pellet to the microbial loop in chalk streams. In this

experiment I investigate the numbers of bacteria associated with *G. pulex* faecal pellets under both laboratory and field conditions. I hypothesised that, in the laboratory, bacterial numbers would increase rapidly during the first few days after pellet egestion, and a similar process would be observed in the field. Bacterial numbers would then remain high as the faecal material is broken down.

4.2.2. Method

Overview

Direct counts of bacterial cells were used to enumerate bacterial numbers on, and within, *Gammarus pulex* faecal pellets incubated both in a laboratory and field experiment. In direct counting bacteria in a sample are stained with a fluorescent dye which allows bacteria to be seen clearly, and numbers counted under a fluorescence microscope. Direct counting is the most accurate form of bacterial enumeration, giving better results than other methods such as counting colony-forming units, which is dependant on culture conditions and even distribution of bacteria on agar plates (Fry 1988).

Faecal pellets were disrupted in a known volume of autoclaved water to suspend the bacteria and other organic materials from the pellet. DAPI solution was then added to stain the bacteria. The resulting suspension was filtered through a 0.22 µm pore size black polycarbonate membrane (Whatman CycloporeTM) which was then mounted onto a microscope slide and viewed under a fluorescence microscope – the bacteria fluorescing brightly against the dark background of the filter membrane. A digital camera recorded high resolution images of the bacteria on the membrane and ImageJ was used to count the

number of bacteria in each image, and consequently on each membrane. This allowed direct counts of the number bacteria in each faecal pellet to be obtained.

Preliminary Staining of Escherichia coli and Faecal Pellets Using DAPI

The fluorescent stain 4',6-diamidino-2-phenylindole or DAPI was selected as the bacterial stain for these direct counting experiments. Acridine Orange (a common nucleic acid stain) has routinely been used in direct counting, but has recently been replaced by DAPI in many studies as it is much brighter when bound to bacteria and does not stain non-target materials (such as detrital aggregates, exopolymers or other biological materials) to a great degree (Porter and Feig 1980, Fry 1988). To test DAPI as a bacterial stain, pure colonies of *E. coli* (provided by David Gems, Department of Biology, UCL) were stained using 1 mg l^{-1} DAPI solution for 30 minutes at 10°C , and photographed under a microscope. Staining was carried out at 10°C to prevent decomposition of the stain as DAPI is temperature sensitive (Fry 1988).

Bacteria appeared as bright blue particles under fluorescence microscopy using a DAPI wavelength filter (Zeiss filter set 02 – gated 365 nm excitation, long pass 420 nm emission) and were easily distinguished. Detrital materials (such as that from faecal pellets) stain poorly with DAPI as it primarily stains DNA. To test this, freshly egested faecal pellets were stained in 1 mg l^{-1} DAPI solution for 30 minutes at 10°C . Pellets were then squashed on microscope slides and observed under fluorescence illumination. Detrital materials from the pellet stained poorly giving a low yellowish glow if at all, compared to the bright blue colour of bacterial cells. Bacteria can therefore be easily distinguished from any detritus present in a stained faecal pellet.

Staining Faecal Pellet Associated Bacteria Using DAPI

To count numbers of bacteria in faecal pellets, samples of pellets were taken, stained and counted. Each sample consisted of 3 *G. pulex* faecal pellets collected using a sterile disposable plastic pipette (all pipettes used were sterile plastic 5 ml 'Liquipettes' – Elkay Èireann, Ireland). The size (as apparent surface area or ASA – see Section 3.4.) of these pellets was measured by placing them on a sterile glass microscope slide using a plastic pipette, and photographing them using a video camera linked to a PC. The images were analysed with ImageJ (see Appendix B and Section 3.4.2.) to give the ASA of each of the 3 pellets in the sample in mm². These measurements were stored for later use.

All 3 faecal pellets in the sample were then disrupted to release the bacteria into suspension for staining. A large sterile cover slip was placed over the pellets, along with a drop of autoclaved water, and gently agitated until the material and bacteria dispersed into the water. The suspension was then washed into a sterile disposable test tube using approximately 10 ml of autoclaved water and a plastic pipette. The slide and cover slip were washed thoroughly to remove all the materials into the water. 1.5 ml of DAPI solution (aqueous) at a concentration of 10 mg l⁻¹ was added to the resulting suspension of bacteria and organic matter for bacterial staining. The volume of the suspension was made up to 15 ml with autoclaved water giving a final DAPI concentration of 1 mg l⁻¹. The suspension was left to stain for 30 minutes at 10°C.

The solution was filtered onto a Whatman CycloporeTM 0.22 µm pore-size polycarbonate membrane, 25 mm in diameter and factory-stained black to avoid background fluorescence. This membrane was held in a 30 ml capacity filter head (Figure 4.2.1.) that was attached to a Buchner flask and pump to provide suction to filter the

sample. Membranes were mounted on top of a Whatman GF/F (glass fibre) 25mm diameter filter paper and glass sinter. The sinter produced even dispersion of suction from the Buchner pump, leading to even distribution of bacteria on the filter membrane, essential for accurate counting (Fry 1988). The filter discs (polycarbonate membrane, GF/F filter and glass sinter) were sealed into the filter head using a pair of Viton rubber sealing rings.

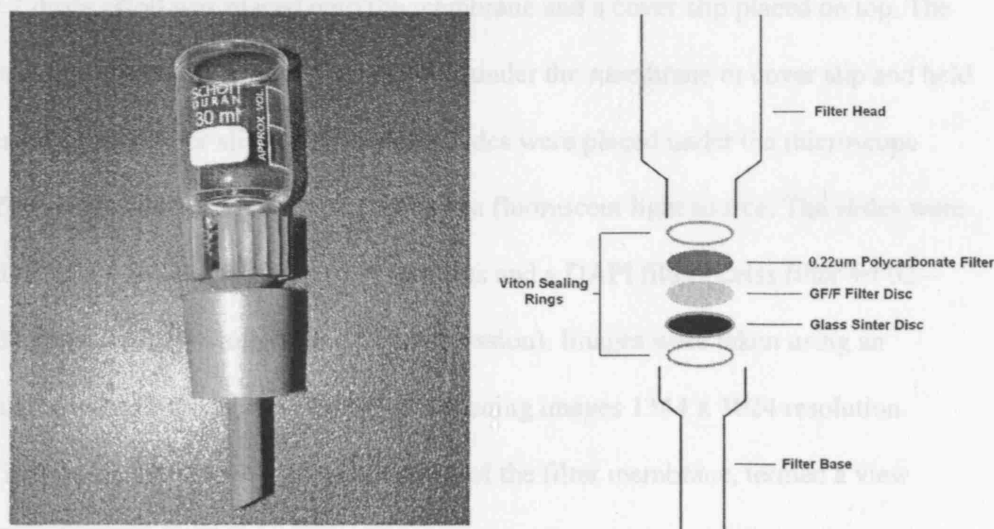


Figure 4.2.1. – Left - filter head used to filter bacteria onto the polycarbonate membrane. Right – arrangement of components within the filter head.

Once the filter head was set up, the faecal pellet + bacteria suspension was poured in and drawn through the filter membrane by the Buchner pump, collecting the stained bacteria and organic matter from the faecal pellet on the membrane. As advised in Fry (1998), excess stain was washed from the membrane to prevent background fluorescence by passing two 15 ml washes of autoclaved water through the filter head. Following this,

membranes were removed and left to dry at 10°C for 45 minutes before being mounted for counting under a fluorescence microscope.

Counting Bacterial Numbers Using Fluorescence Microscopy

Dry membranes were mounted on microscope slides by placing 2 drops of non-fluorescent immersion oil onto the slide's surface and placing the membrane on top. A further 2 drops of oil was placed onto the membrane and a cover slip placed on top. The immersion oil prevented air becoming trapped under the membrane or cover slip and held the membrane and cover slip onto the slide. Slides were placed under the microscope (Carl Zeiss Axioskop 2) and illuminated using a fluorescent light source. The slides were viewed using a 40x magnification objective lens and a DAPI filter (Zeiss filter set 02 – gated 365 nm excitation, long pass 420 nm emission). Images were taken using an trinocular attached Hamamatsu Orca-ER, producing images 1344 x 1024 resolution.

Each image taken was a view of a small area of the filter membrane, termed a view field. To gain accurate direct counts of bacteria it is important to count as many random view fields as is feasible (Jones and Simon 1975, Hobbie *et al.* 1977). Using Microsoft Excel, I developed a random co-ordinate generator which returned random positions on the membrane to take images for counting. A total of 25 random view fields were photographed in this way from each membrane for counting.

Quantification of Bacterial Numbers Using Image Analysis

ImageJ was used to count the images taken of each membrane (see Appendix B). This method is quick and accurate and commonly used in direct counts of bacteria in water

and sediment samples (Sieracki *et al.* 1985, Kuwae and Hosokawa 1999). To make sure that using ImageJ was as accurate as counting the images manually, a short experiment was conducted to compare ImageJ counts with manual counts.

A faecal pellet sample was prepared and filtered as described above, and 25 view fields photographed. These view fields were counted by two human subjects in different rooms with no contact with one another – myself and a project student from our laboratory. These human counts were compared to the results of counting the same images with ImageJ. Mean counts made by myself and the project student were 80.27 (\pm SD 40.89) and 87.27 (\pm SD 38.65) bacterial cells per view field respectively, and 91.50 (\pm SD 46.33) bacterial cells per view field by ImageJ. When compared statistically we found no significant difference between counts made by either human subject and those made by ImageJ ($p = 0.388$ and 0.750 respectively in t-test). Image analysis therefore is just as accurate as human counting the numbers of bacteria on each membranes.

The images taken of each membrane were counted automatically by ImageJ, and the numbers of bacteria in each image recorded for analysis. From these counts, a mean number of bacteria per image (from 25 images in total) was calculated. In a trial experiment where 5 samples of faecal pellets were analysed, coefficients of variation ranged from 13.71% to 27.27%.

By multiplying the average number of bacteria in a view field by the total number of view fields needed to cover the whole membrane, an estimate of the number of bacteria in the 3 original faecal pellets was produced. Finally, using the ASA measurement of the faecal pellets, the number of bacteria per mm^2 of faecal pellet ASA was calculated and this was used as a measure of bacterial numbers.

Considerations For Interpretation of Bacterial Numbers

Although data collected here from both the laboratory and field contains important information about bacterial numbers in *G. pulex* faecal pellets, the numbers themselves are probably under estimates of the true absolute numbers. Many bacteria were hidden under particles of organic matter from the faecal pellet matrix, lowering the counts made in both lab and field experiments. Coarse filter paper has been used successfully to remove such particles before filtering onto the polycarbonate membrane to give more accurate counts of bacteria, but because pellets contain abundant EPS (see Section 3.2.) this technique was not used as it was feared that many bacteria would become stuck in the filter paper. The counts obtained were experimentally valid however because the experiment was primarily concerned with comparisons (over time, and between stream habitats), and because every sample was treated the same way. This problem therefore does not influence the overall conclusions of the experiments.

The Laboratory Experiment : Faecal Pellet Production and Incubation

G. pulex faecal pellets were produced from laboratory cultures fed on conditioned Horse Chestnut leaves. *G. pulex* were left to feed for 24 hours and the pellets produced were collected on a sieve, forming an experimental stock of pellets with an age range of 0 to 24 hours. The stock was suspended in 1 litre of chalk stream water (all chalk stream water used was from the River Chess) using a magnetic stirrer, and this suspension split into 5 x 200 ml aliquots, each with the same amount of faecal material in suspension, and these placed into 5 beakers. The pellets were left to settle in these beakers for 1 hour and after this time most of the water was drawn off using a pipette (leaving ~20 ml of pellets and

water). Each aliquot was then transferred to a 50 ml plastic sample container for incubation and chalk stream water added so the total volume was 50ml. The lids to the containers had air holes cut into them to allow gas exchange with the atmosphere and they were incubated at 10°C (the temperature at which water enters a chalk stream; Berrie 1992). These formed 5 replicates which were sampled 0, 1, 2, 5, 13, 20, 30, 34, 37, 41, and 51 days after the start of the experiment and the numbers of bacteria they contained counted.

The Field Experiment : Faecal Pellets in Different Habitats

To complement the laboratory experiment, a field experiment was conducted. Pellets were produced from laboratory cultures of *G. pulex* fed on conditioned Horse Chestnut leaves. A pellet stock suspension was produced as above for the laboratory experiment and 10 x 100 ml aliquots (each containing the same number of faecal pellets) were placed into beakers to settle for 1 hour. Excess water being drawn off using a pipette (leaving ~20 ml of pellets and water). Each aliquot was then transferred to a 50 ml plastic sample container for transport to a field site where the pellets were placed into traps in the substratum, effectively incubating them under environment conditions.

The faecal pellets were placed into artificial substrate traps embedded in the substratum of the River Chess. The traps used were very similar to those used in the experiments in Section 2.3., but had a lid made from 200 µm mesh placed on them when in the stream. This mesh prevented loss of pellets, deposition of fresh pellets in the trap and coprophagy by invertebrates. They however allowed water, dissolved gases and micro-organisms to enter the trap.

A field site 100 m downstream from that used in 2.3. was chosen for the traps as it had both marginal and mid-channel vegetation, and areas of stream channel with no vegetation. Traps were placed in areas of stream channel without vegetation, under marginal macrophyte stands and under emergent *Ranunculus* stands in mid channel. This was repeated at 3 sites approximately 25 metres apart downstream, giving 9 traps in total (3 clear channel, 3 marginal vegetation, 3 *Ranunculus*). Traps were implanted so they were flush with the substratum, and easily removed and replaced because of a wire mesh lining to the hole they were placed in (see Section 2.3.2.).

At the start of the experiment, each trap was stocked with the faecal pellets from one of the 10 aliquots produced from the pellet stock (9 aliquots were used in total, 1 being left as a spare). Once stocked the mesh lid placed on the trap and it was put into the substratum of the river. A sample of faecal pellets from each trap was taken after 0, 1, 7, 15, 21, 28, 36, and 45 days and returned to the laboratory for bacterial counting. During the experiment, stream water temperature was monitored and mean water temperature was 19°C, almost identical to the temperature used in the laboratory incubation.

4.2.3. Results

Bacterial Numbers of Laboratory Faecal Pellets

Gammarus pulex faecal pellets incubated in the laboratory showed a peak in bacterial numbers 1-2 days after the start of the experiment, and a smaller peak later on after 37 days (Figure 4.2.2.). These results suggest that bacterial numbers increase quickly soon after the faecal pellet has been egested, and again later in the life of the faecal pellet after bacteria have had time to break down the organic materials in the pellet.

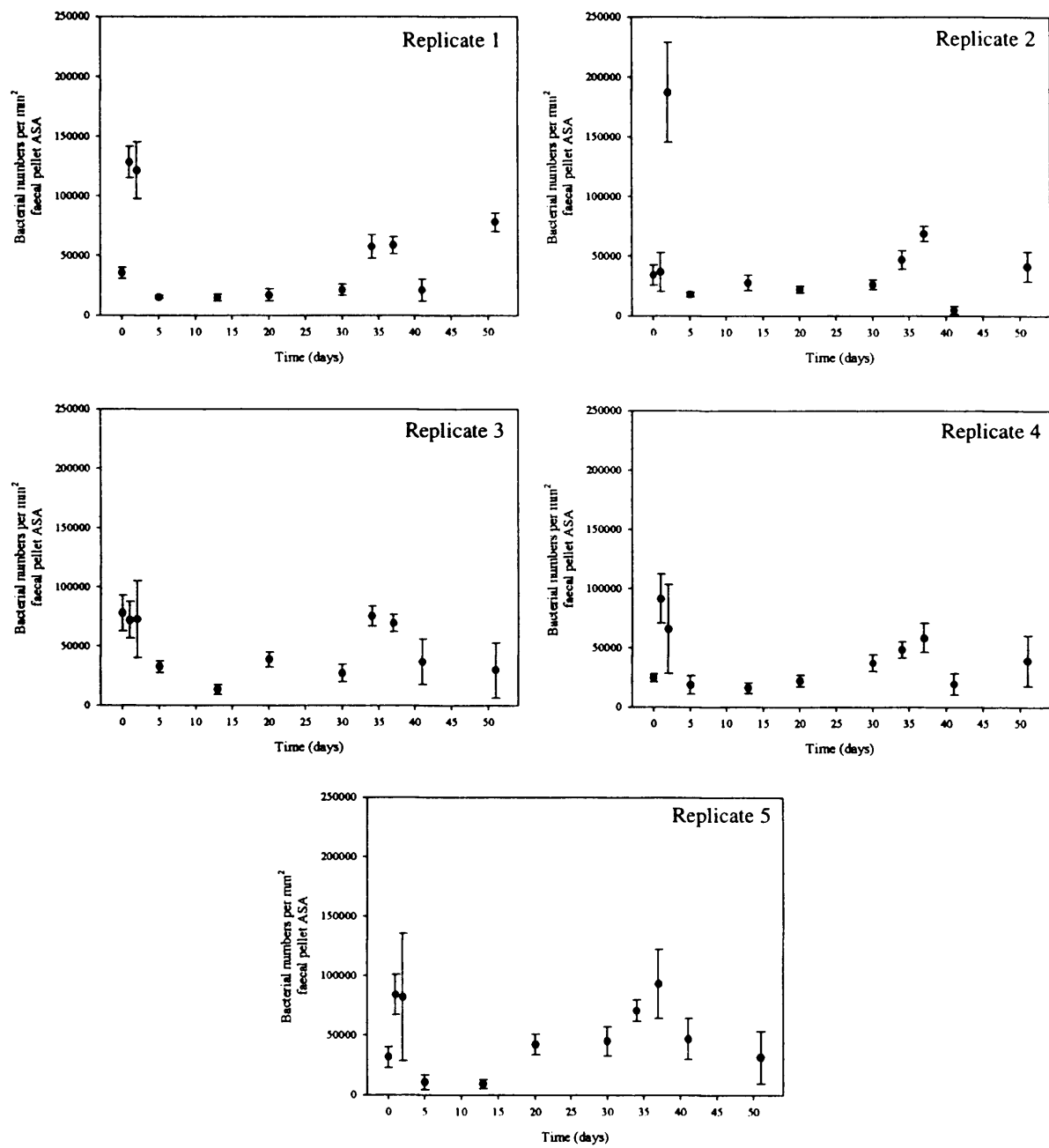


Figure 4.2.2. – Bacterial Counts over 51 days from 5 replicate groups of *G. pulex* faecal pellets incubated at 20°C in the laboratory. Whiskers indicate standard error of the mean.

Bacterial Numbers of Faecal Pellets in the Field

Bacterial numbers on, and within *G. pulex* faecal pellets placed in all 3 chalk stream habitats (clear channel, marginal macrophyte stands, *Ranunculus* stands) showed a similar pattern of breakdown to those incubated in the laboratory (Figure 4.2.3.). Bacterial numbers peaked after 3 days indicating that bacteria also grow quickly on faecal pellets in the field soon after egestion. In pellets placed under marginal macrophytes and in clear channel there was no distinct second peak however, as after ~25 days bacterial numbers became highly variable and showed no recognisable pattern of growth and decay (Figure 4.2.3. A and B). Under *Ranunculus* however there was a steady rise in numbers to 21 days after being placed in the stream, and after this they began to fall again (Figure 4.2.3. C). There was no statistical difference between the bacterial numbers in all three treatments ($F_{2,54} = 0.630$ $p = 0.562$ in repeated measures ANOVA). Conditions within the pellets at egestion allow bacterial numbers to increase rapidly, whereas later in the life of the pellet bacterial numbers become more variable, but relatively low compared to the initial peak in numbers.

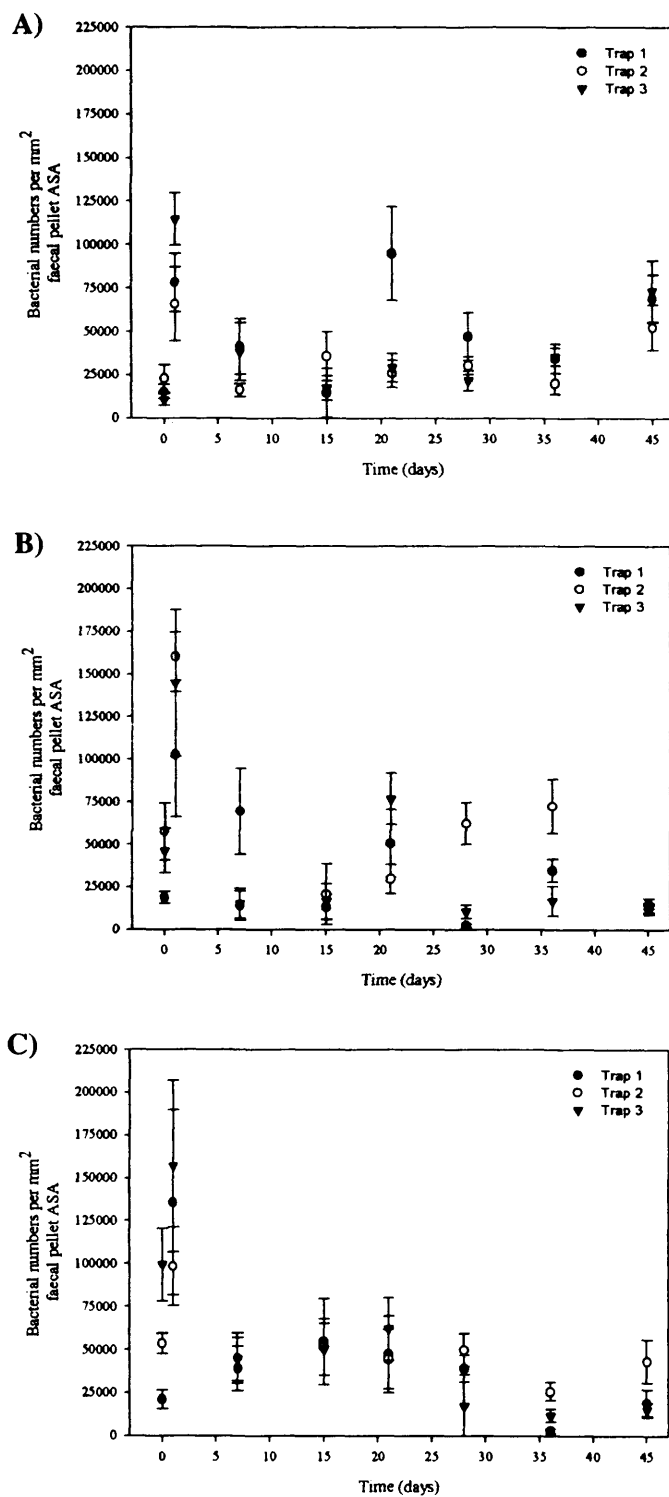


Figure 4.2.3. – Bacterial counts over 45 days from faecal pellets placed A) in clear stream channel (no vegetation), B) under marginal vegetation and C) under *Ranunculus* stands. Whiskers indicate standard error of the mean.

4.2.4. Discussion

In both laboratory and field treatments, *Gammarus pulex* faecal pellets showed a peak of bacterial abundance after 2-3 days. Similar increases in bacterial number have been described repeatedly in freshly egested marine zooplankton faecal pellets (Jacobsen and Azam 1984, Gonzalez and Biddanda 1990, Povero *et al.* 2003, Olsen *et al.* 2005), and it is highly likely to be related to the availability of DOM. Invertebrate faecal pellets leach DOM soon after egestion (Jumars *et al.* 1989, Urban-Rich 2001), and bacterial metabolism and reproduction increases quickly as a reaction to the presence of these readily available labile substrates (Thor *et al.* 2003, Olsen *et al.* 2005). Once all the DOM has leached or been absorbed, bacterial numbers decrease quickly due to a lack of available substrates causing many bacteria to die. This may explain the early peak in bacterial numbers observed for *G. pulex* faecal pellets in the laboratory and field experiments. Because highly active bacteria become bound into faecal pellets (see Section 3.4.), they are surrounded by DOM and other labile materials, reproducing quickly on this resource. However, because the material cannot sustain large numbers of bacteria for more than 2-3 days break down of the faecal pellet matrix is slow and pellets are relatively stable and cohesive, retaining their structure for long periods. As shown in Sections 3.4. and 3.5., pellet ASA increases substantially over the first 2-3 days after the egestion of the pellet, presumably because the large numbers of bacteria release exoenzymes which break down the bonds within the pellet matrix. Bacterial numbers decrease after this time and pellet ASA changes little for the following 30 days because their low numbers have little effect on pellet structure. This means that pellets are relatively stable for 30-35 days. After this time microbial breakdown processes increase

in significance and the pellet matrix breaks down (shown by the increases in ASA observed in Section 3.5.), and this is probably linked to the second peak in bacterial abundance seen in the laboratory experiments.

Although at the start of the experiments both laboratory and field faecal pellets showed a similar pattern of bacterial growth, the pellets left in the field showed no distinct second peak of growth as in the laboratory pellets because bacterial numbers became highly variable. Pellets incubated in the laboratory are not subject to the more complex microbial communities present in the field. The numbers of bacteria associated with the pellets placed in the field were probably influenced by environmental conditions and colonising micro-organisms such as protozoa which feed on bacteria (Sleigh *et al.* 1992). Habitat type did not influence the overall numbers of bacteria in *G. pulex* faecal pellets over the 45 days they were sampled however, and therefore different habitats do not increase or decrease microbial colonisation or growth on faecal pellets. Bacterial numbers on, and within, *G. pulex* faecal pellets are not influenced by the environment upon their egestion (as internal bacteria are present), but over time (after ~30 days) the environment begins to influence the numbers of bacteria which become highly variable.

4.3. Microbial Respiration in *Gammarus pulex* Faecal Pellets

4.3.1. Introduction

Bacteria and many other micro-organisms utilise DOM and POM from invertebrate faecal pellets as a substrate, breaking down and metabolising the organic matter within them (Olsen *et al.* 2005). In streams and rivers, DOM is an important microbial substrate (Meyer and O'Hop 1983, Meyer 1994) but little attention has been given to the POM stored within faecal pellets, potentially a significant resource for micro-organisms.

Organic matter bound into faecal pellets is mineralized more quickly by micro-organisms than non-bound organic matter in both streams and oceans (Jones 1995, Turner 2002), and the microbial community within pellets differs in structure from the community in the wider environment (Hansen *et al.* 1996). Faecal pellet production therefore has significant effects on mineralization of organic matter in aquatic environments, and affects how organic matter is passed to successive trophic levels. In chalk streams, where the numbers of pellets per unit area is relatively high (Ladle and Griffiths 1980), faecal pellets are likely to be important centres of microbial metabolism and recycling of organic matter.

In this experiment the respiration of micro-organisms living on and within *G. pulex* faecal pellets was investigated. I hypothesised that the rate of respiration of the microbial community of faecal pellets would be relatively high and show an increase over time in a similar pattern to those seen in bacterial numbers in Section 4.2.

4.3.2. Method

Production of an Experimental Faecal Pellet Stock

Gammarus pulex faecal pellets were produced from laboratory cultures using Horse Chestnut leaves as food. *G. pulex* were left for 24 hours to produce pellets and after this time pellets were collected by pouring the water in the culture tanks through a 100 μm sieve on which the pellets were retained. They were then washed using tap water (de-chlorinated by bubbling) into a single 2 litre beaker, and 1 litre of de-chlorinated tap water was added, producing an experimental stock of faecal pellets. The pellets were suspended in the water column using a magnetic stirrer on low power.

Measurement of the Number and Mass of Faecal Pellets in the Experimental Stock

The number of faecal pellets in the experimental stock was then counted (in order to calculate the mass of material present) using volumetric sub-sampling of the suspended faecal pellets. Using a 1 ml plastic pipette, a 1 ml sample of the suspension was taken and counted under a binocular microscope using a Sedgewick-Rafter cell (1 ml capacity). This was repeated 20 times and the average number of faecal pellets per ml of the experimental stock was calculated, giving a value of 74.60 (\pm SD 2.79) pellets per ml. 250 ml of de-chlorinated tap water was added to the experimental stock to bring the concentration of faecal pellets to \sim 100 per ml, and the stock was counted again as above. The final concentration of faecal pellets in the experimental stock was 117.23 (\pm SD 32.02) pellets per ml of suspension

The mass of material in the experimental stock was also estimated. A further 20 samples were taken from adjusted stock suspension as above, placed in a Sedgewick-Rafter cell (1

ml capacity) and photographed using a high resolution (8 mega pixel) digital camera. The total ASA (see Section 3.4. to 3.6.) of faecal pellets in each sample was measured using ImageJ and the values stored for analysis.

Larger faecal pellets have a greater mass than smaller faecal pellets, as shown in Section 2.2.4. Using these data, the relationship between faecal pellet size and mass was calculated, giving a factor allowing conversion of faecal pellet ASA measurements in mm^2 to mass in mg. The 20 ASA measurements taken from the experimental stock were therefore converted to measurements of mass in mg ml^{-1} .

Incubation of the Experimental Stock and Sample Extraction

After counting and mass estimation, the experimental stock was split into 5 replicates and each incubated at 20°C for the duration of the experiment. Samples were taken from each replicate by re-suspended it using a magnetic stirrer, and a sterile plastic pipette used to extract 5 ml of the suspension. The sample was transferred to an oxygen electrode so that the respiration of micro-organisms associated with the pellets could be measured. The stirrer was switched off as soon as a sample had been taken to reduce the amount of physical disturbance of the faecal pellets in the stock. By this method, the concentration of pellets in each replicate was maintained throughout the duration of the experiment.

Measurement of Microbial Respiration Using an Oxygen Electrode

The consumption of oxygen, and therefore rate of respiration, by the microbial community in the *G. pulex* faecal pellets was measured by using an oxygen electrode. A Rank electrode was chosen for this as the apparatus is easy to assemble and maintain,

reliable and most importantly can be sealed to prevent diffusion of oxygen in from the environment. This makes it possible to record the consumption of oxygen by micro-organisms living on and in detrital material (such as faecal pellets) over time (Hitchman 1978)

The apparatus (Figure 4.3.1.) consists of a pair of electrodes (a platinum cathode and silver anode) immersed in 3M potassium chloride solution and separated from the sample under investigation by an oxygen permeable Teflon membrane. Oxygen diffuses through the membrane and the redox reaction occurring at the electrodes creates a voltage which is proportional to the concentration of oxygen in the sample solution. The electrodes, potassium chloride and membrane are positioned on the base of the apparatus

Figure 4.3.1. – Diagrammatic representation of the Rank oxygen electrode or Clark cell (image copyright Rank Brothers Ltd.).

with the sample incubation chamber located on top. A stopper seals the incubation chamber to prevent environmental oxygen from diffusing in, and during measurements the sample solution is kept constantly mixed by a magnetic stirrer.

As metabolic rate of bacteria and other micro-organisms is linked closely to temperature, a water jacket around the incubation chamber acts as a thermostat. This jacket was fed by an aquarium pump from a constant temperature water bath set at 20°C, and the sample solution was kept at 20°C during experimental measurements of oxygen consumption. Measurements were recorded by a chart recorder connected to the apparatus. The electrode was calibrated at the beginning of every day using a small amount (~10 mg) of sodium dithionite which removes all oxygen from solution and allows the recording range between 0% and 100% oxygen to be set.

To measure the respiration in a sample of faecal pellets, 5 ml of each replicate (~500 faecal pellets) was pipetted into the incubation chamber, which was then sealed and the apparatus allowed to equilibrate for 1 minute. The concentration of oxygen in the sample was then monitored for 1 hour. After this time the recording was stored for analysis and the chamber washed thoroughly with distilled water ready for the next sample.

Experimental Treatments

Faecal pellets in each replicate were sampled for their oxygen consumption after 1, 2, 4, 7 and 14 days (the critical period of bacterial growth identified in Section 4.2.). To control the experiment, a 50 ml aliquot of each replicate was taken at the start of the experiment and autoclaved to destroy the microbial community within the pellets, halting respiration. The pellets were then placed into fresh (unautoclaved) stream water and the

oxygen consumption measured in the electrode. The 5 replicates were tested as described above, and no consumption of oxygen was detected (oxygen consumption by suspended micro-organisms was too low to be detected), indicating that any oxygen consumption seen was due to microbial respiration within pellets and not from chemical oxidation or micro-organisms suspended in the chalk stream water.

4.3.3. Results

Quantification of the Faecal Pellet Stock

The relationship of *Gammarus pulex* faecal pellet ASA with mass was positive and linear (Figure 4.3.2.), with every mm² of pellet ASA equalling 0.126 mg of faecal material (dry mass). From sampling the experimental stock suspension it was determined that 1 ml of stock contained 117.23 (\pm SD 32.02) *G. pulex* faecal pellets, amounting to 1.87 (\pm SD 0.52) mg of material (Table 4.3.1).

Microbial Respiration Over Time

All the experimental incubations of *G. pulex* faecal pellets consumed oxygen demonstrating active microbial respiration within the faecal pellets (Figure 4.3.3.). There was no significant difference in the rate of respiration of faecal pellets over time ($H = 3.25$ $p = 0.354$ in Kruskal-Wallis test). The rate of microbial respiration occurring inside faecal pellets was therefore roughly constant during the time they were incubated.

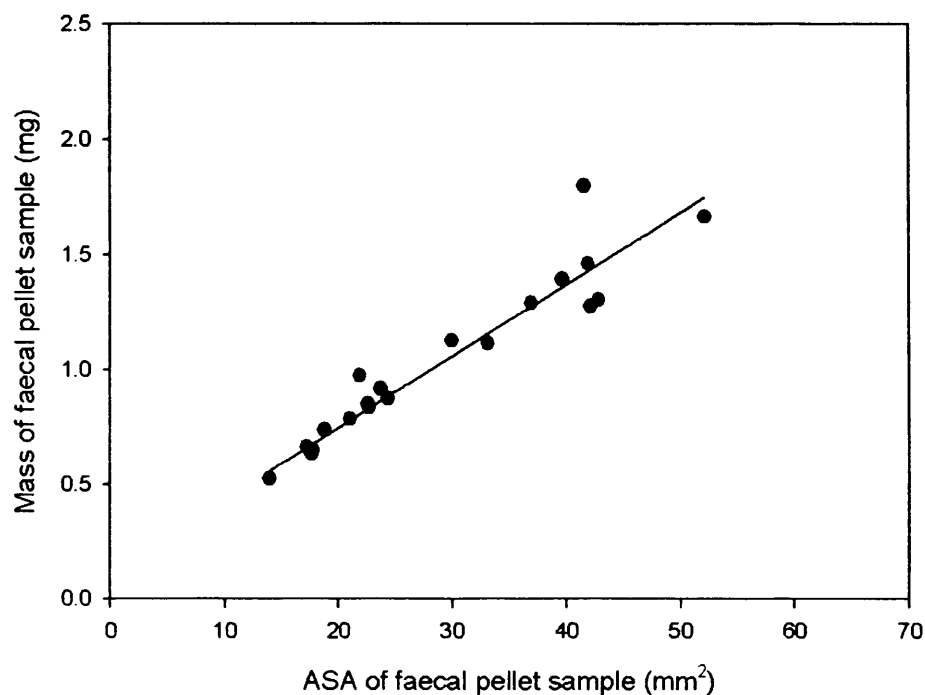


Figure 4.3.2. – Plot and regression line showing the relationship of faecal pellet ASA with faecal pellet mass ($y = 0.1263 - 0.0311x$, $r^2 = 0.9362$, $n = 20$, $p < 0.001$), 1 mm² of faecal pellet ASA equals 0.126 mg dry mass of faecal material.

Mean number of <i>Gammarus</i> faecal pellets in ml of stock suspension	Mean ASA (mm ²) of these pellets	Conversion factor to mass (mg dry mass)	Mean mass (mg dry mass) of <i>Gammarus</i> faecal pellets in 1 ml of stock suspension
117.23 (± SD 32.03)	14.85 (± SD 4.17)	0.126	1.87 (± SD 0.52)

Table 4.3.1. – Quantification of the *G. pulex* faecal pellet stock suspension used in experiments.

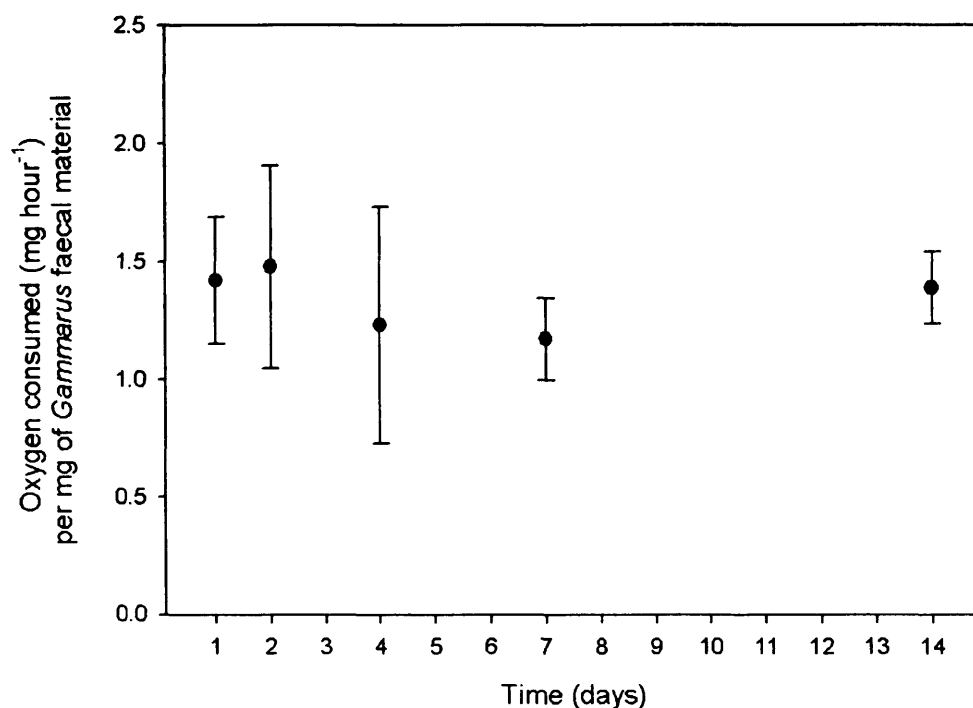


Figure 4.3.3. – Changes in mean oxygen consumption ($n = 5$) of *G. pulex* faecal material over 14 days of incubation (whiskers equal the standard deviations of mean oxygen consumption).

4.3.4. Discussion

Micro-organisms on, and within, *Gammarus pulex* faecal pellets metabolise organic matter from the pellets themselves, utilising it as a substrate. Faecal pellets are therefore a source of carbon for the micro-organisms in chalk streams, and are important to the recycling of organic matter in these systems.

No significant change in respiration rate was observed over the 14 day incubation period of the faecal pellet stock, which is surprising as the numbers of bacteria in pellets show a peak 1 - 2 days after egestion (see Section 4.2.). Because of this a similar increase in respiration rate was expected to account for the increased metabolism of the additional bacteria, but this did not occur, and respiration rate remained more or less constant over

the course of the experiment. The peak in bacterial numbers may represent anaerobic bacteria (which do not consume oxygen) and as such an increase in their numbers is not detected by measurements of respiration.

Respiration rate is high compared to microbial communities on other forms of stream and river detritus. Hill et al (1992) reports respiration rates of ~ 0.001 to ~ 0.008 mg O₂ mg⁻² leaf hour⁻¹ from prairie streams in North America, 3 orders of magnitude lower than observed on *Gammarus* faecal pellets where respiration rate was ~ 1.2 to 1.5 mg O₂ mg⁻² faecal pellet hour⁻¹. Microbial respiration in *G. pulex* pellets is also high when compared to sediments. In their review of microbial respiration rates from sediments in 196 North American streams, Hill et al. (1998) report respiration rates ranging from 0.00005 to 0.00016 mg O₂ mg⁻² sediment hour⁻¹. However, anaerobic respiration may dominate in sediments where oxygen penetration is low and partly explain the low respiration rates observed in these studies. The aerobic microbial community within faecal pellets is stimulated to much higher rates of activity than microbial communities utilising other aquatic detritus. As mentioned previously, bacterial activity is stimulated by passage through protistan food vacuoles (Atlas 1999). Passage through the gut of *G. pulex* probably greatly increases the rate of metabolism of bacteria and other micro-organisms mixed into the pellet, so that when the pellet is egested, microbial respiration is high.

Because of their relatively high respiration rate when compared to other forms of stream detritus, *G. pulex* faecal pellets can be considered hotspots of microbial respiration, as zooplankton faecal pellets are in the oceans (Alldredge and Cohen 1987, Tang 2005). The organic matter within them is respired at a relatively high rate making a significant contribution to the recycling of organic matter in chalk streams when compared to other

forms of detritus. In sediments especially, faecal pellets increase the depth oxygen can penetrate and as aerobic respiration is more efficient than anaerobic metabolism, enhance the turnover of organic matter (Wild *et al.* 2005).

4.4. Utilisation of *Gammarus pulex* Faecal Pellets by Chalk Stream Invertebrates

4.4.1. Introduction

Higher animals (both vertebrate and invertebrate) gain nutrition from faecal material – the process of feeding by coprophagy. Rabbits and other members of the Leporidae are a well understood case as they lack both the ability to breakdown cellulose from their diet, and a rumen that localises symbiotic bacteria (as in many Artiodactyls) that will breakdown the cellulose. Coprophagy not only provides a mechanism of re-inoculating the gut with cellulose degrading bacteria but also allows a second pass of the material through the gut to gain further nutrition (Hirakawa 2001). Termites also feed in this way (Nalepa *et al.* 2001). Feeding by coprophagy can be advantageous for an organism where other sources of food are in short supply, and/or an abundance of high quality faeces are available (Fenolio *et al.* 2006).

Coprophagous organisms also occur in aquatic ecosystems where they recycle organic matter not assimilated and egested by other organisms (Frankenberg and Smith 1967). Blackfly larvae often collect faecal pellets as they filter particles from the water column and this has been shown to be an advantageous feeding tactic (Wotton 1980b), and zooplankton in lakes feed by coprophagy and significantly reduce the flux of organic matter to the bottom of the lake (Pilati *et al.* 2004). Neither of these studies indicate that faecal pellets are chosen over more regular food sources however, and it is likely that the majority of faecal pellets are consumed incidentally or as a supplement to the diet when higher quality food is not available (Brendelberger 1997). Freshwater gastropods also

feed by coprophagy, but this is mainly to gain further nutrition from material where one pass through the gut is not enough (Brendelberger 1997).

The abundance of faecal pellets in chalk streams represents a large standing crop of organic matter (and therefore a resource) available for chalk stream invertebrates and micro-organisms to utilise. As there is a high abundance of detritivores in these streams (Pardo and Armitage 1997) it is likely that coprophagy is a widespread feeding strategy, recycling large amounts of organic matter from faecal pellets both in suspension and in sediments.

Using the hypothesis that other chalk stream detritivores consume faecal pellets, I investigated coprophagy by other invertebrates on *G. pulex* faecal pellets. My objective was to show whether or not organic matter in *G. pulex* faecal pellets is utilised by other chalk stream organisms as a food resource.

4.4.2. Method

Fluorescent Labelling of Gammarus Faecal Pellets

Gammarus pulex faecal pellets were produced that contained fluorescent paint particles in order to track their consumption by other invertebrates. These paint particles (from Radiant Color[®], California, USA) do not harm the invertebrate and pass through the gut unchanged (Wotton 1992, Wotton *et al.* 1998). *G. pulex* were fed conditioned Horse Chestnut leaves that had been incubated in a suspension of paint particles for 2 - 3 days. The suspension was kept constantly mixed using an automatic laboratory stirrer so that the surface of the leaves was evenly coated with paint particles, and as biofilm developed on the surface of the leaves the paint particles were trapped. These leaves were fed to

laboratory *G. pulex* instead of untreated leaves, and egested faecal pellets contained paint particles mixed into the pellet. These were termed 'labelled' pellets. Labelled pellets were collected from culture tanks by passing the water through a 200 µm sieve and stored in plastic 25 ml vials at 10°C until needed.

Experimental Animals and Treatments

Asellus sp. (Crustacea : Isopoda), *Ephemera* sp. (Insecta : Ephemeroptera) and *Planorbis* sp. (Mollusca : Gastropoda) were collected from the River Chess, Buckinghamshire (see Figure 2.3.1. & 2.3.2.) and kept in the laboratory in an aerated plastic aquarium tank (25 cm length x 15 cm width x 15 cm height) filled with dechlorinated tap water until needed for experiments. These organisms are all common chalk stream detritivores.

Previously unused (and exposed to fluorescent particles) aquarium tanks identical to those described above, and containing no faecal pellets were populated with either 10 *Asellus*, 10 *Ephemera* or 10 *Planorbis* (organisms of each taxon were never mixed). Each of these treatments was replicated 4 times. Labelled *G. pulex* faecal pellets were added to each tank, along with a 2 cm x 2 cm piece of unlabelled conditioned Horse Chestnut leaf as an alternative food, and the organisms were left for 24 hours to feed. After this time they were transferred to a fresh tank containing clean water with no label and no food. They were left for a further 24 hours to egest faecal pellets (termed 'experimental faecal pellets'). These were collected from each tank using a disposable pipette and analysed to quantify the amount of label they contained.

Assessment of coprophagy

A total of 10 experimental faecal pellets were taken from each experimental tank, squashed on microscope slides and observed under a fluorescence microscope. The fluorescent particles were clearly visible if present. Digital photographs of the total amount of label present were taken and quantified using ImageJ (see Appendix B). A score was awarded based on the mean amount of label observed in the 10 pellets : -

- \times : $< 0.01 \text{ mm}^2$ label observed (no significant feeding on faecal pellets);
- \checkmark : $> 0.01 \text{ mm}^2$ label observed (significant feeding on faecal pellets).

We used this threshold to eliminate possible errors as some paint particles may have been released from pellets into the water of the experimental tanks and taken up directly by organisms. In preliminary experiments, we found that this source of contamination amounted to $\sim 0.001 \text{ mm}^2$ of observable label in faecal pellets.

4.4.3. Results

All three experimental taxa (*Asellus*, *Ephemera* and *Planorbis*) fed on *Gammarus pulex* faecal pellets as fluorescent label was found in the experimental faecal pellets they egested (Table 4.4.1.). No label was found in experimental faecal pellets from two of the replicates (1 & 2) conducted with *Ephemera*, probably because the *Ephemera* in these tanks did not feed, as they produced very few faecal pellets compared to the other *Ephemera* replicate tanks (3 & 4).

Organism	Replicate 1	Replicate 2	Replicate 3	Replicate 4
<i>Asellus</i> sp.	✓	✓	✓	✓
<i>Ephemera</i> sp.	×	×	✓	✓
<i>Planorbis</i> sp.	✓	✓	✓	✓

Table 4.4.1. – Feeding by *Asellus*, *Ephemera* and *Planorbis* on *Gammarus* faecal pellets. Key : × = no significant feeding on faecal pellets (< 0.01 mm² of label detected), ✓ = significant feeding on faecal pellets (> 0.01 mm² of label detected).

4.4.4. Discussion

Chalk stream detritivores feed by coprophagy on *Gammarus pulex* faecal pellets. Although the test used in the above experiment is purely qualitative, showing that detritivores consume faecal material, it indicates that they may digest and assimilate the materials from the pellet transferring energy directly from faecal pellets. There is evidence from the literature that *G. pulex* faecal pellets are a significant nutritional resource as young *G. pulex* feed and grow on them when in laboratory culture (McCahon and Pascoe 1988a, McCahon and Pascoe 1988b). Also, when in the brood pouch young *Gammarus* show evidence of feeding on organic matter from the parent's feeding behaviour (Welton *et al.* 1983), and it is likely that organic matter from the parent's faecal pellets are also consumed.

Coprophagy may be an important energy transfer mechanism in chalk streams with many invertebrate detritivores regularly consuming faecal pellets, enriched in nutrients by bacterial growth (Shepard and Minshall 1981). Marine copepods often feed by coprophagy resulting in a significant increase in the amount of energy recycled in the photic zone and increasing productivity there (Frankenberg and Smith 1967). There are few studies of coprophagy by freshwater organisms, but those that do exist suggest it is widespread (Wotton *et al.* 1998) and the results of this experiment suggest that this is also true for chalk streams.

4.5. General Discussion

4.5.1. Utilisation of *Gammarus* faecal pellets in chalk streams

Gammarus pulex faecal pellets are a food source both for micro-organisms and invertebrate detritivores in chalk streams. Bacteria and other elements of the faecal pellet microbial community actively metabolise organic matter from pellets, using it to grow and reproduce. The microbial loop therefore recycles large amounts of organic matter in chalk streams because the high respiration rate of the micro-organisms on and in faecal pellets is relatively high. Microbial production is probably high on and in faecal pellets providing a basis for consumer communities.

Bacteria are particularly important as they are consumed in large numbers by protozoans which are abundant in chalk streams (Sleigh *et al.* 1992). Bacteria are also rich in protein which enhances the nutritional value of faecal pellets (Shepard and Minshall 1981). As well as utilisation by micro-organisms, *G. pulex* faecal pellets are consumed by a variety of chalk stream invertebrates which presumably gain nutrition from them and the abundant microbial community within the pellet in a similar situation to the utilisation of dead leaves by shredders (Cummins 1974).

4.5.2. Patterns of microbial growth and utilisation

Bacteria are the most important micro-organisms involved in *G. pulex* faecal pellet breakdown in chalk streams (as shown in Section 3.4.), and this is true of faecal pellets in other aquatic environments such as the oceans (Hansen *et al.* 1996). In *G. pulex* faecal pellets, bacterial numbers increase quickly soon after egestion in response to DOM and labile organic materials within the pellet matrix (Olsen *et al.* 2005). But as no

corresponding increase in respiration rate was observed the bacteria increasing in number (and utilising the DOM) must be anaerobes. The anaerobes are probably opportunistic species that reproduce quickly in order to utilise labile DOM before it leaches out of the pellets (Jumars et al 1989), and subsequently they have no substrate to feed on and start to die. Opportunistic bacteria are therefore lost early from the pellets, leaving species that can utilise more refractory substrates (which pellets are rich in). This leads to a relatively constant and high rate of respiration from the surviving microbial community within pellets, but relatively low numbers of bacteria when compared to their initial abundance.

The early peak in bacterial growth is likely to be important to invertebrates feeding on faecal pellets. Bacterial growth adds protein to the pellets as it does to allochthonous materials (Cummins 1974, Shepard and Minshall 1981, Sanzone *et al.* 2001), and the initial increase in bacterial numbers (and their subsequent decline) probably acts as a large input of protein to the pellet matrix which increases their nutritional value to coprophagous detritivores and micro-organisms. *G. pulex* faecal pellets are therefore stores of high nutritional value material that can be utilised by a variety of organisms within the stream.

4.5.3. Coupling terrestrial organic matter and chalk stream organisms by faecal pellets

As described previously, faecal pellets represent a significant transformation of allochthonous organic matter in chalk streams, shunting this material from leaf packs to sediments. Because coprophagy is practised by a variety of chalk stream detritivores (see Section 4.4.), allochthonous materials become more widely distributed among chalk stream communities due to the production of faecal pellets by *G. pulex*. Non-shredder

organisms can consume and access the allochthonous organic matter not assimilated by *G. pulex* and egested in their faecal pellets by consuming whole pellets or particles released from their breakdown. For example, filter feeders such as *Simulium* cannot access the allochthonous materials in dead leaves directly, but can consume allochthonous organic matter particles released from *G. pulex* faecal pellets and suspended in the current of the stream. The terrestrial inputs from leaf litter are therefore more effectively coupled to chalk stream communities because of the production and presence of *G. pulex* faecal pellets in sediments, and allochthonous materials can be utilised by a wider range of organisms than would otherwise be possible.

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5. General Discussion

5.1. A Theoretical Model of the Role of *Gammarus pulex* Faecal Pellets in Chalk Streams

The faecal pellets of *Gammarus pulex* are egested into the substratum and sediments of chalk streams. Pellets are bound strongly by EPS inside the pellet matrix, and initially by a peritrophic membrane which degrades quickly after egestion. Because of this strong binding, pellets are long lived and remain cohesive for long periods (80 days or more) at normal stream temperatures. When temperatures increase to 20°C, fragmentation of pellets begins at around 30 days after egestion, suggesting that when water temperatures in the stream increase (such as in summer), the rate of pellet degradation increases and fragmentation is promoted. Degradation of pellets is primarily caused by bacteria internal to the pellet matrix and these bacteria transform the organic matter there over time, creating larger aggregate particles of organic matter (SecAggs) from the fine materials present at egestion. This occurs because of changes in binding of materials inside the pellet by EPS secreted from micro-organisms. These transformations have important implications for how organic matter from faecal pellets is transported and retained in

chalk streams. *G. pulex* faecal pellets are consumed by invertebrates and utilised by micro-organisms of the microbial loop (particularly bacteria) as a substrate and form an important ecological link in chalk streams.

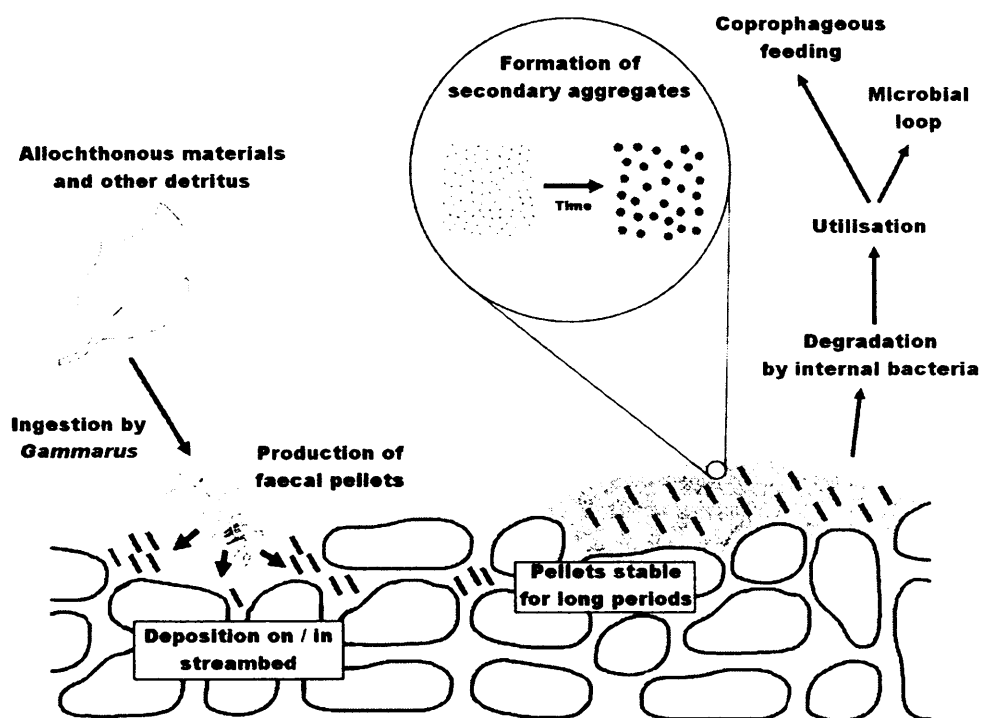


Figure 5.1.1. – Theoretical model of *G. pulex* faecal pellet production, transformation and degradation in a chalk stream.

Faecal pellets are a much overlooked component of stream ecosystems. They are important as stores of allochthonous organic matter (a significant energy source for chalk streams and other lotic ecosystems), sites of organic matter transformation and decomposition, and a food source both for invertebrates and micro-organisms. Faecal pellet formation is known to alter organic matter transport in streams and rivers by altering particle size (Wotton and Malmqvist 2001), but the results presented here show

that *Gammarus pulex* faecal pellets and the organic matter they contain are altered by microbial processes long after they are egested. These processes promote organic matter retention within the stream, especially allochthonous materials which are pelletised by *Gammarus* feeding on dead leaves. The faecal pellets of *G. pulex* remain intact in sediments, and are then available for other invertebrates to utilise.

Micro-organisms also utilise this material and play a significant role in transferring it to higher trophic levels. I suggest that the production of faecal pellets by *G. pulex* is an important recycling process for organic matter in chalk streams, and probably in other stream and river systems as well. Faecal pellets must therefore be considered in greater detail when studying energy flows in streams and rivers. Future work should focus on other stream systems in temperate zones other than chalk streams, and stream systems in are climatic zones such as the tropics, to ascertain to contribution faecal pellets play to organic matter transfer in these systems. It is likely to be high given data already obtained for chalk streams and Swedish rivers. In chalk streams future work would ideally focus on using tracers to track the movement of faecal pellet organic matter such as looking at which communities utilise most faecal material (invertebrate, microbial or both) and to which communities the material is passed to first, and in what amounts.

5.2. Faecal Pellets and the Ecology of Streams and Rivers

Although invertebrates consume faecal pellets, the most significant decomposers of faecal pellet organic matter appear to be micro-organisms. Bacteria are the principle agents of breakdown of *Gammarus pulex* faecal pellets and they also cause a change in the size of organic matter particles within faecal pellets. This transformation ultimately

leads to enhanced retention of organic matter within the stream, and the productivity and overall amount of energy in the chalk stream ecosystem is increased.

Invertebrates are the most *visible* detritivores in chalk streams and other flowing waters, but bacteria are the main decomposers of organic matter. This was first shown by Odum (1957) and has been confirmed since in other systems such as lake outlets (Wotton 1988). Marxsen (2006) recently showed that bacteria are the main decomposers in the Breitenbach stream in Germany, accounting for 59% of total respiration, and 78% of the heterotrophic respiration. Bacterial respiration is enhanced on faecal pellets in marine systems (Thor *et al.* 2003), and respiration rates of the microbial community of *G. pulex* faecal pellets are relatively high when compared to rates quoted for stream sediments in the literature (see Section 4.3.4.). As a result, pelletisation of organic matter by invertebrates is likely to increase the rate of break down of organic matter by bacteria. This will in turn increase the nutritional value of sediments, as bacterial production adds protein to the pellets, making them a more attractive food resource for invertebrates.

5.3. Perspectives on the Role of Faecal Pellets in Aquatic Environments

Chalk streams are productive environments with lush plant growth and high biomasses of invertebrates and fish (Berrie 1992). Faecal pellets are likely to be a key resource for chalk streams due to their stability (pellets remain intact for long periods), abundance (pellets are constantly being produced, deposited and transported), and because they are a food substrate for both invertebrates and bacteria (see Chapter 4). Chalk stream sediments are highly pelletised, with a significant proportion of the total organic matter being in the form of faecal pellets (Ladle and Griffiths 1980). *Gammarus pulex* pellets

are mainly found within the substratum (see Chapter 2), but large deposits also build up under macrophyte stands (Cotton *et al.* 2006), and it is possible that pellets in both these areas supply nutrients for plant growth.

The pelletisation of a sediment has a significant effect on its physical features. Polychaete worms feeding in coastal sediments cause those sediments to become pelletised and this has a range of effects including an increase in permeability of the sediment to gases (e.g. oxygen) by two orders of magnitude, and increased porosity which causes a higher through-flow of water in the sediment (Wild *et al.* 2005). As *G. pulex* faecal pellets are much larger than fine particulate organic matter in chalk stream sediments, similar processes are likely to occur. For example in between stream bed pore spaces fine organic matter may fill pore spaces and prevent water movement whereas faecal pellets only roughly pack together and give large spaces for water and oxygen movements. Also, in Australian mangrove swamps where the dominant shredders are typically crabs, conversion of organic detritus (in the form of dead mangrove leaves) to faecal pellets increases the rate of organic matter mineralization, and this in turn enhances the transfer of energy to higher trophic levels (Werry and Lee 2005). In these two cases pelletisation enhances the turnover of organic matter in sediments, and as a result more energy is released from detritus and passed to higher trophic levels. It is highly likely that similar processes occur in chalk streams, increasing organic matter turnover in pelletised sediments under *Ranunculus* stands and enhancing the transfer of energy. It is important to note however that pelletisation of organic matter is a product of invertebrate activity, and as such will be determined by the populations of invertebrates available to produce faecal pellets. Because drought events significantly lower the abundance of invertebrates,

particularly that of *G. pulex* (Wood and Armitage 2004), the numbers of faecal pellets being produced and the amounts of organic matter being converted into faecal pellets significantly reduced. It is unclear how this will affect the ecosystem, but it is likely that a variety of plant, invertebrate and microbial communities dependant on detritus within the stream will be affected. This area certainly deserves more attention.

G. pulex are important as they convert large amounts of allochthonous organic matter from dead leaves in leaf packs to faecal pellets in autumn, and these pellets are deposited in sediments, shunting organic matter from terrestrial sources into the sediments. This may provide an important and slowly released source of energy to the chalk stream ecosystem during winter when levels of productivity are low and plants die back.

Faecal pellets in chalk streams not only store organic matter but also transport it.

Simulium faecal pellets are found frequently in the water column and form a significant flux of organic matter downstream where pellets are deposited in areas of low flow such as under *Ranunculus* stands (Warren *et al.* 2005, Cotton *et al.* 2006). Organic matter is transported as whole or partial faecal pellets resuspended from sediments, and also particles released from pellets that have broken down. Transformation of these particles, particularly in size but also in other physical features such as porosity, influences the downstream flux of organic matter. Over time, particles within *G. pulex* faecal pellets undergo significant transformation which increases their size, and this reduces the distance they are transported downstream by increasing their sinking rate. Such transformations, mediated by bacteria, influence the transport of organic matter in chalk streams on a large scale.

Although the body of literature describing faecal pellets in streams and rivers is expanding, they have often been ignored, and as such very few papers focus on faecal pellets. Especially lacking is data from streams and rivers in climatic zones other than temperate northern areas. Grimm (1988) suggested that that faecal pellets in hot desert streams are important in nitrogen recycling as they are “an abundant and reliable high-quality food resource” for invertebrates, and Yule (1996) hinted that the detritus in aseasonal tropical streams is dominated by faecal pellets due to the presence of large numbers of Simuliid larvae and a predominance of fine particulate organic matter, which is similar to the situation in chalk streams.

The small number of freshwater papers on faecal pellets is in contrast to the marine literature which has widely investigated the role of faecal pellets in a variety of systems all over the globe. Since the discovery by Turner and Ferrante (1979) that the majority of sinking organic particles in the ocean are faecal in origin, a great deal of attention has been paid to faecal pellets, particularly those egested by copepods. Oceanographers have developed advanced methods which are not often employed by freshwater biologists, due in part to a lack of dialogue between the disciplines (Wotton 2007). In the oceans it is appreciated that the transfer of organic matter by faecal pellets is a complex process, featuring both physical, chemical and biological factors that vary over time and space (Turner 2002). As a result, there are many areas where the marine literature about faecal pellets can help us understand how they function in freshwater systems, and there is a need for greater attention to be paid to faecal pellets in streams and rivers across a variety of climatic zones so their role can be fully understood. Factors affecting *Gammarus* faecal pellets in chalk streams are of a similar complexity to those occurring in marine

systems, but ultimately there are differences, as faecal pellets are stored in chalk streams and rather than constantly produced and utilised or lost to the sea bed as in the oceans.

Future research into organic matter transfer in streams and rivers will require not only an appreciation of the potential role of faecal pellets, but a more thorough integration of different scientific disciplines and methods, as has occurred in marine biology over the past two or three decades. Faecal pellets of *G. pulex* and other invertebrates are a significant ecological component of chalk streams, mediating the storage, transfer and release of organic matter as well as providing a food source to a wide variety of micro-organisms and invertebrates. This is likely to be true for many other stream and river ecosystems around the world, and if the role of faecal pellets is studied in detail we may soon be able to answer some of the important questions about the ecology of our flowing waters. Alternatively, if the subject is ignored, we will only make slow progress towards a more complete understanding of streams and rivers.

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Appendix A – Quantification of Protein in Dead Leaf Food

Introduction

Nutritional value of allochthonous detritus is typically determined by protein content, the higher the protein content the higher the nutritional value of the detritus (Cummins and Klug 1979). Foods of high nutritional value can be termed high 'quality' foods. The quality of diet of aquatic invertebrates can affect both faecal pellet characteristics (e.g. size) and transport (e.g. sinking rate) and is therefore an important consideration (Griffin 2000). This experiment demonstrates that dead Horse Chestnut (*Aesculus hippocastanum*) leaves are relatively high in protein and dead English Oak (*Quercus robur*) leaves are relatively low in protein, and these species therefore constitute high and low quality diets respectively.

Methods

Overview

The Bradford's dye binding (also known as Coomassie Brilliant Blue) method was used to quantify the protein content of dead leaves. Leaves were collected in October of 2003 from autumn leaf fall and kept in a freezer until needed in experiments. Leaves were used as food for laboratory *Gammarus*. Two different species were chosen – Horse Chestnut (*Aesculus hippocastanum*) and English Oak (*Quercus robur*). The former is preferred by *Gammarus* in laboratory culture (McCahon and Pascoe 1988) and assumed to have a high protein content. The latter is not preferred and is assumed to have a low protein content. The experiment tests this assumption.

Reagents

- pH 9.0 Bicine buffer 200 mM (Bicine + KOH)
- 10% SDS
- 1M Dithiothreitol (DTT)
- Polyvinylpyrrolidone (PVPP) powder
- Bradford's Reagent – Sigma-Aldrich premixed

Leaf Disc Preparation

Leaf discs were cut from dead leaves of both species using a 1 cm diameter cork borer. Each disc was taken from a different leaf. Leaves were taken from frozen stocks described above. Discs were weighed on a microbalance and the weight of each disc recorded. Discs were then placed in a mortar for protein extraction. A total of 10 replicate discs were sampled per species.

Protein Extraction

Each leaf disc was treated separately. Individual leaf discs were ground in a pestle and mortar with 270 µl Bicine buffer, 30 µl 10% SDS and 1.5 µl DTT to extract the protein. A small amount of PVPP was also added to prevent any tannins present from binding to protein and preventing detection. The resulting extract was centrifuged for 3 minutes to sediment out the solid materials and supernatant removed by pipette. This was further centrifuged for 3 minutes and supernatant removed to an Eppendorf tube.

Protein Quantification

A 10 μl sample of protein extract was mixed in a micro-cuvette with 200 μl of Bradford's Reagent and mixed by inversion. The absorbance of the resulting solution was read at 495 nm in a spectrophotometer. Absorbance was converted to mg ml^{-1} of protein using a standard curve prepared using serial dilutions of Bovine Serum Albumin (BSA). This concentration allowed calculation of the total amount of protein in each leaf disc, and extrapolation to the amount of protein per gram of leaf.

Results

Horse Chestnut leaves contained on average $13.20 \text{ mg protein g leaf}^{-1}$, whereas Oak leaves contained half this at $7.64 \text{ mg protein g leaf}^{-1}$. This is shown in Figure A.1.

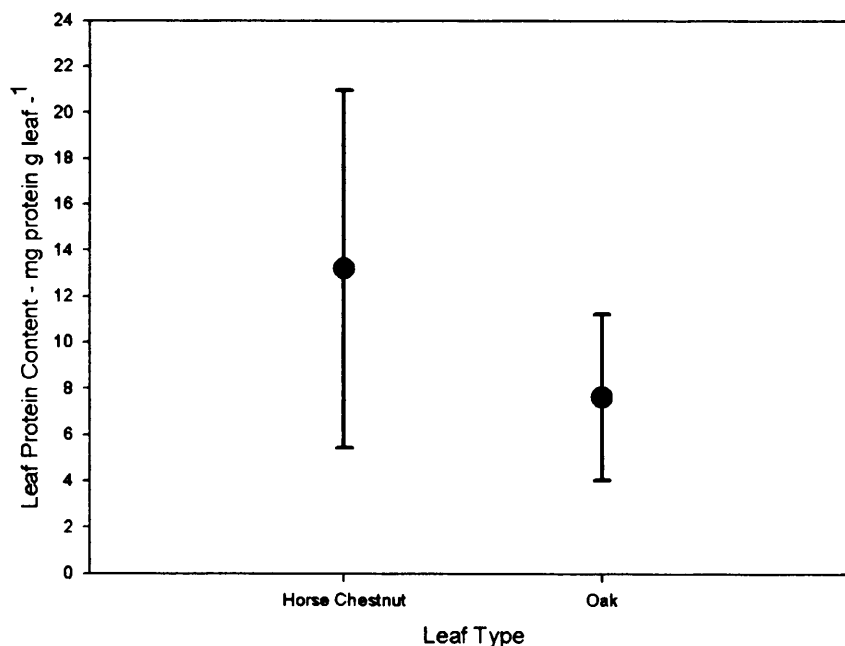


Figure A.1. – Plot showing the mean protein content of Horse Chestnut and Oak leaves (whiskers indicate \pm standard deviation). Oak leaves can be considered a low quality diet due to their relatively low protein content. Horse Chestnut leaves are a high quality diet due to their relatively high protein content.

Because Oak leaves have a relatively low protein content, they can be considered a low quality diet. Conversely Horse Chestnut leaves can be considered a high quality diet because they have a relatively high protein content.

Discussion

Horse Chestnut leaves can be considered a high quality diet and Oak leaves a low quality diet due to their respective protein contents. This allows these two leaves to be used as laboratory standards in experiments where the effect of diet quality is under investigation.

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Appendix B – Basic Particle Sizing and Counting Using ImageJ

Introduction

Digital image analysis is the process of extracting useful measurements from calibrated photographic data held in a computer file. Image analysis is a fast and accurate method of measurement and is used in a wide variety of industrial (Petruck and Skinner 1997, Talbot *et al.* 2000, Zhou *et al.* 2004) medical (Matteson *et al.* 1996) and scientific (Kuwaie and Hosokawa 1999, Gering and Atkinson 2004) applications. Although specialist image analysis systems have been available for biological research since the 1980s, these systems were relatively large, inflexible and difficult to use (see the equipment used in Sieracki *et al.* 1985). Modern image analysis can be carried out by anyone on a personnel computer quickly and easily, and the results immediately available. With the advent of free, open-sourced image analysis software such as ImageJ (US National Institutes of Health), the scientific applications of image analysis have greatly expanded. ImageJ is a powerful and easy to use image analysis tool and is the software I used in all my experiments involving image analysis.

One of the most basic and useful functions that ImageJ and other image analysis packages is the counting and size measurement of ‘particles’ within the image. A particle is a discrete object within the image (e.g. in an image of a human blood cells each cell is considered a particle) and ImageJ will count and measure the size of all particles in the image (for blood cells, the total number of cells and the size of each).

We used image analysis primarily to count and measure the size of faecal pellets, but it was also used to count bacteria within faecal pellets (Section 4.2.), measure the size of

secondary aggregates formed within faecal pellets (Section 3.3) and determine whether faecal pellets themselves form aggregates (Section 3.7.). In this appendix, the process of particle counting and size measurement using ImageJ is discussed.

Image Capture Equipment

We used three different types of equipment to capture digital images in our experiments, each was selected on the basis of each experiment's requirements.

- a) **CCTV camera with 50mm lens attached to a PC (Figure B.1.A)** – Using a video capture card and software linked to a standard CCTV camera it was possible to capture digital images of subjects. Images were of good resolution (720x576 pixels), and this combined with the 50mm lens mounted onto the camera (which gave a close-up view of subjects in view) meant a large amount of information was captured in each image. This equipment was mostly used to measure the size of *Gammarus* faecal pellets over time (e.g. Sections 3.4. and 3.5.).
- b) **High resolution digital camera (Figure B.1.B)** – A Canon EOS 350D digital camera with a macro lens was used to gain high resolution (3456x2304) images of small particles, such as those released from disrupted faecal pellets (Section 3.3).
- c) **Zeiss microscope with digital camera attached (Figure B.1.C)** – This equipment was used to view microscopic subjects such as fluorescent tracer particles within faecal pellets (Section 4.4.) and DAPI stained bacteria for direct counting (Section 4.2.). The images were of high resolution (1344x1024) and

contained a high level of information which facilitated accurate measurements of microscopic subjects.

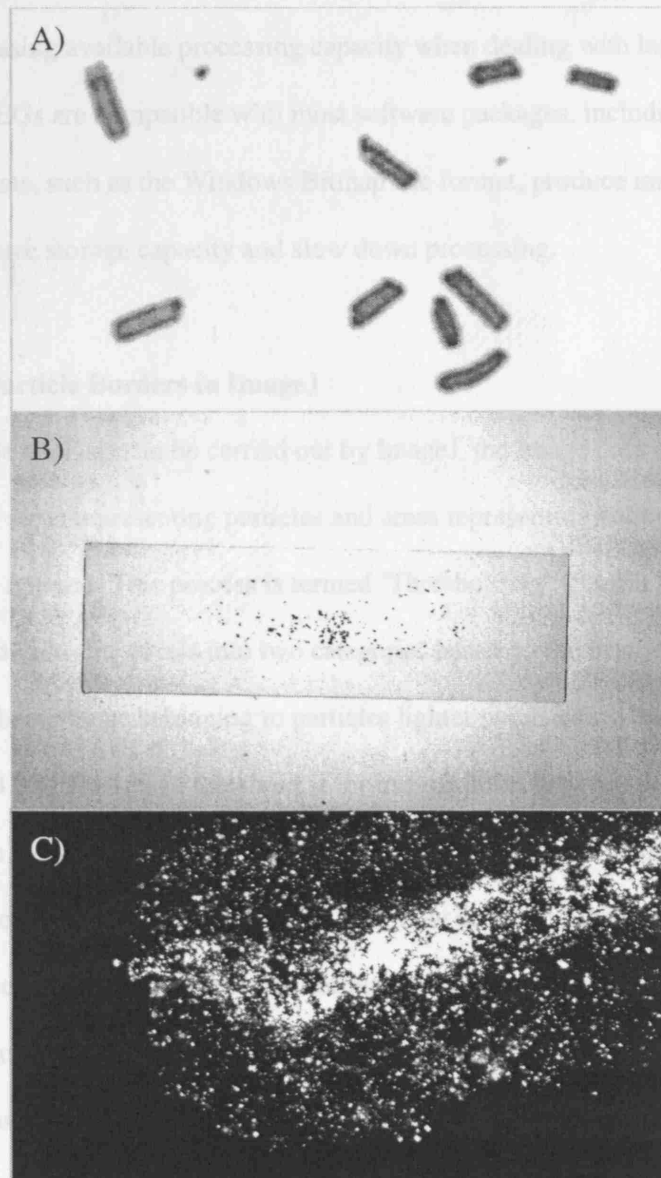


Figure B.1. – Example images from the three types of capture equipment used in experiments. Images show A) *Gammarus* faecal pellets captured using a CCTV camera with 50mm lens connected a PC, B) *Simulium* faecal pellets on a slide taken using a high resolution digital camera with macro lens, and C) fluorescent tracer particles inside a *Gammarus* faecal pellet taken using an epifluorescence microscope mounted digital camera.

Image File Storage

Images were converted to the JPEG (Joint Photographic Experts Group) format after creation. This format is easy to work with as file sizes are very small (conserving storage space and increasing available processing capacity when dealing with large numbers of images) and JPEGs are compatible with most software packages, including ImageJ. Other image file formats, such as the Windows Bitmap file format, produce much larger image sizes which reduce storage capacity and slow down processing.

Determining Particle Borders in ImageJ

Before particle analysis can be carried out by ImageJ, the image data needs to be partitioned into areas representing particles and areas representing background elements which are to be ignored. This process is termed 'Thresholding' (Sezgin and Sankur 2004). ImageJ divides the pixels into two categories based on the brightness of each pixel - darker pixels being those belonging to particles lighter pixels being background elements (Fig B.2.). The image threshold is the cut-off point between these categories. When an image has had a threshold applied it is converted into a 2 colour binary image – black particles on white background. ImageJ can now count and measure the size of individual particles as the pixels have an definite border.

Thresholds can be determined manually or automatically by ImageJ. Automatic thresholding has the advantage over manual thresholding as it is based on mathematical equations and not biased by human opinion. ImageJ uses the isodata algorithm to determine thresholds automatically (Ridler and Calvard 1978).

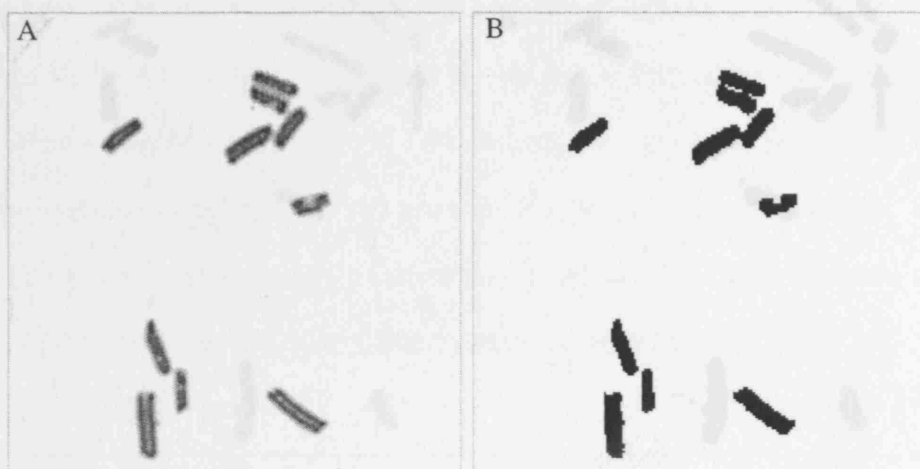


Figure B.2. – Shows a standard 256 greyscale image capture of *Gammarus* faecal pellets from a CCTV camera (image A) and the same image after automatic thresholding in ImageJ (image B). In A no definite edges are defined so the size and number of particles cannot be determined. In B particles are defined as they have a definite edge (where black particles meet white background) and image analysis can proceed.

However, where background elements are clearly visible (such as the sides of Petri dishes, reflections from lights) these may give an incorrect threshold and misleading measurements (Fig B.3.).

Our image capture equipment and experimental methods were setup to greatly reduce this problem. Where possible, edges of containers and other interfering structures were not included in the images by repositioning the camera, or removed by digitally cropping the images. If despite these precautions the automatic threshold was incorrect, then one was determined manually, although this was very rare.

Particle Counting

After thresholding an image ImageJ can analyse and measure the particles it contains, and particle counting is the simplest measurement. ImageJ looks for discrete particles and

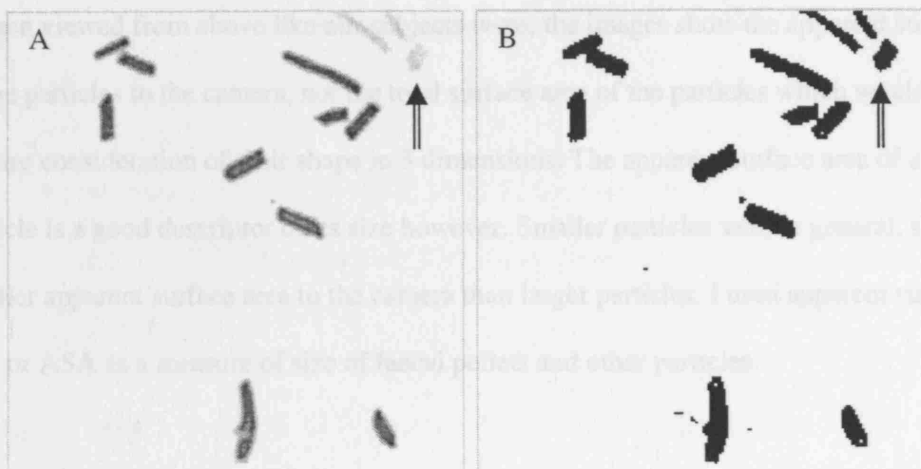


Figure B.3. – Shows a demonstration of errors that can occur with binary thresholding. Image A is of faecal pellets from a CCTV camera capture and image B is the same image after binary thresholding in ImageJ. In A there is a reflection of a group of 3 faecal pellets on the edges of the Petri dish containing the faecal pellets, shown by the arrow. This reflection has been included by ImageJ in the thresholded image B and will cause an error in the number and total area of particles in the image.

will output the number in the image. Data was saved in Microsoft Excel format and then analysed.

Particle Size – Apparent Surface Area as a Measurement of Particle Size

As well as counting particles in a image, ImageJ can measure their 2-dimensional area. The software determines the number of pixels in a particle, and refers to a predetermined calibration value which tells it each pixel is equivalent to an area in mm^2 , μm^2 or whatever spatial scale is being used to calibrate the image. I calibrated each piece of equipment before use using a microscope micrometer slide (used to calibrate eyepiece graticules in microscopes). Particle sizes were generally calculated in mm^2 and saved in Microsoft Excel format as above.

When viewed from above like our subjects were, the images show the apparent surface of the particles to the camera, not the total surface area of the particles which would require consideration of their shape in 3 dimensions. The apparent surface area of a particle is a good descriptor of its size however. Smaller particles will, in general, show a smaller apparent surface area to the camera than larger particles. I used apparent surface area or ASA as a measure of size of faecal pellets and other particles.

Discussion

Particle counting and size measurement using ImageJ is a fast and accurate method of analysis. Although the system has limitations, such as errors in automatic threshold calculation, I designed the method to reduce these problems. Using ImageJ greatly increased both accuracy of measurements and work efficiency as image capture is a relatively easy and quick task compared to more traditional methods such as measurement of faecal pellet size under a microscope. Image analysis is also versatile and I applied it to a wide variety of experimental situations.

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